# Active Matrix Metalloproteinase-8 Chairside Oral Fluid Test in Association with Subclinical Periodontitis, Dental Caries, and Health Behaviour in Finnish Adolescents

**Teija Raivisto**

**University of Helsinki**
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Department of Oral and Maxillofacial Diseases
Faculty of Medicine
University of Helsinki
Helsinki, Finland

# ACTIVE MATRIX METALLOPROTEINASE-8 CHAIRSIDE ORAL FLUID TEST IN ASSOCIATION WITH SUBCLINICAL PERIODONTITIS, DENTAL CARIES, AND HEATH BEHAVIOUR IN FINNISH ADOLESCENTS

Teija Raivisto

ACADEMIC DISSERTATION

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**Supervised by:**
Docent Anna Maria Heikkinen
University of Helsinki, Finland
Department of Oral and Maxillofacial Diseases
Helsinki University Hospital, Helsinki, Finland

Professor Timo Sorsa
University of Helsinki, Finland
Department of Oral and Maxillofacial Diseases
Helsinki University Hospital, Helsinki, Finland
Division of Periodontology, Department of Dental Medicine
Karolinska Institutet, Huddinge, Sweden

**Reviewed by:**
Professor emerita Vuokko Anttonen
University of Oulu, Finland
Department of Cariology, Endodontology and Pediatric Dentistry
Research Unit of Oral Health Sciences

Professor Arzu Tezvergil-Mutluay
University of Turku, Finland
Department of Restorative Dentistry and Cariology
Senior Consultant, Turku University Hospital
Adhesive Dentistry Research Group Leader

**Opponent:**
Professor Satu Lahti
University of Turku, Finland
Department of Community Dentistry
Centre for Population Health Research

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To my family

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**ABBREVIATIONS**

| | |
|---|---|
| *A.actinomycetemcomitans* | *Aggregatibacter actinomycetemcomitans* |
| aMMP-8 | Active matrix metalloproteinase-8 |
| AUC | Area under the curve |
| BOP | Bleeding on probing |
| CAL | Clinical attachment loss |
| CI | Confidence interval |
| CPITN | Community periodontal index of treatment needs |
| D | Decayed tooth |
| DMF | Number of decayed, missed and filled permanent teeth |
| DNA | Deoxyribonucleic acid |
| ECM | Extracellular matrix |
| ELISA | Enzyme-linked immunosorbent assay |
| FDR | False discovery rate |
| GCF | Gingival crevicular fluid |
| $H_2O_2$ | Hydrogen peroxide |
| HELENA | Healthy Lifestyle in Europe by Nutrition in Adolescence |
| HOCl | Oxidant hypochlorous acid |
| i | Initial caries |
| IFMA | Immunofluorometric assay |
| IFN-γ | Interferon gamma |
| IL | Interleukin |
| IQR | Interquartile range |
| LT-α | Lymphotoxin-alpha |
| LPS | Lipopolysaccharide |
| *LTA* gene | Lymphotoxin-alpha gene |
| MHC | Major histocompability complex |
| MMP | Matrix metalloproteinase |
| MMP-8 | Matrix metalloproteinase-8 |
| MPO | Myeloperoxidase |
| NE | Neutrophil elastase |
| OCl | Hypochlorite |
| OD | Optical density units |
| *P. gingivalis* | *Porphyromonas gingivalis* |

| | |
|---|---|
| PGLYRP1 | Peptidoglycan recognition protein 1 |
| PISF | Peri-implant sulcular fluid |
| PMN elastase | Polymorphonuclear elastase |
| PMN leukocyte | Polymorphonuclear leukocyte |
| POC | Point-of-care |
| PPD | Periodontal pocket depth |
| RC | Root calculus |
| RNA | Ribonucleic acid |
| ROC | Receiver operating characteristic curve |
| ROS | Reactive oxygen species |
| SAAVNA | Succinyl-alanyl-alanyl-valine-nitroanilide |
| *S. mutans* | *Streptococcus mutans* |
| SNP | Single-nucleotide polymorphism |
| sTREM-1 | Soluble triggering receptor expressed on myeloid cells 1 |
| *T. denticola* | *Treponema denticola* |
| *T. forsythia* | *Tannerella forsythia* |
| TIMP | Tissue inhibitors of matrix metalloproteinases |
| TNF-α | Tumor necrosis factor-alpha |
| TNF-β | Tumor necrosis factor-beta |
| TREM-1 | Triggering receptor expressed on myeloid cells 1 |
| VDR | Vitamin D receptor |
| VPI | Visible plaque index |
| WHO | World Health Organization |
| ΔOD405/h | The difference in the optical density units (OD) detected by spectrophotometer at 405 nm before and after 1 hour |

**ABSTRACT**

Periodontitis and dental caries are common chronic diseases, even adolescents suffer not only from caries but also from early stage of periodontitis, subclinical periodontitis. Both diseases are influenced by environmental, host-derived, and genetic factors. Recognition of these factors may reveal susceptibility to disease at an early stage, thus enabling preventive interventions.

Oral fluids contain many potential biomarkers for periodontitis. Matrix metalloproteinase (MMP)-8 and especially its active form aMMP-8 seems to be key biomarkers for periodontitis. The level of aMMP-8 in oral fluids can be measured by the specific aMMP-8 chairside test. The triggering receptor expressed on myeloid cells 1 (TREM-1) together with its ligand peptidoglycan recognition protein 1 (PGLYRP1) are recently recognized salivary biomarkers amplifying proinflammatory processes in periodontal diseases.

The aims were to examine among Finnish adolescents whether the genetic background of subclinical periodontitis and dental caries could be detected (Studies I and II) and to assess salivary levels of PGLYRP1 and its correlation with TREM-1, polymorphonuclear elastase (PMN elastase) and aMMP-8 (study III). Further aims were to investigate whether participants at risk for subclinical periodontitis could be identified using aMMP-8 chairside mouth rinse test and whether treatment and preventive intervention is reflected in the test results (Study IV). The hypotheses of the study were that aMMP-8 levels would be associated with participants' risk for subclinical periodontitis and that treatment and preventive intervention would decrease the aMMP-8 levels. We assumed correlations between proinflammatory biomarkers and expected to find a genetic polymorphism linked to subclinical periodontitis and dental caries.

Studies I-III were carried out at Kotka Health Center in Eastern Finland in 2004-2005 and/or 2014-2015. Participants were 15-17 years old. Altogether 501 participants in 2004-2005 and 47 in 2014-2015 consented to the study. With their permission DNA analyses were performed on 47 participants in 2004-2005 and on all participants (n=47) in 2014-2015. Oral health was examined, including caries and periodontal examination, and whole saliva samples were collected. The aMMP-8 chairside test was performed. DNA was genotyped for 29 candidate genes for periodontitis in Study I and for 26 candidate genes for dental caries in Study II. In Study III, adolescents clustered as periodontally healthy, gingivitis, or subclinical periodontitis were tested for salivary levels of TREM-1, PGLYRP1, aMMP-8, and PMN elastase. Saliva samples were measured by time-resolved immunofluorometric assay (IFMA), by enzyme-linked immunosorbent assay (ELISA) or by synthetic assay (SAAVNA).

Study IV was carried out at the Hämeenlinna Health Center in Southern Finland in 2017-2018 by comparing eighth grade adolescents (aged 14-15 years). Altogether 125 participants consented to the study and the mouth rinse analyses. Adolescents were randomly divided into a control group (n=55) and a test group (n=70). All participants were examined for caries and periodontal status. Participants filled in a questionnaire that consisted of questions concerning health behaviour. Chairside mouth rinse test for aMMP-8 was performed on the test group and analysed by a method for chairside diagnostic testing. The test-positive participants received intensified oral hygiene instructions. Periodontal status and possible change in aMMP-8 test were monitored after intervention.

Participants who were aMMP-8 chairside test-positive and had three or more ≥ 4 mm deepened periodontal pockets showed an association with single-nucleotide polymorphisms (SNPs) in *vitamin D receptor* (*VDR)* and *matrix metalloproteinase* (*MMP)-3* genes. A tendency, but no significant association between SNPs in *DDX39B* and *myeloperoxidase (MPO)* genes and dental caries was found.

Salivary levels of PGLYRP1 and aMMP-8 were significantly higher in adolescents with subclinical periodontitis and gingivitis than in individuals with healthy periodontium. PGLYRP1 correlated positively with bleeding on probing (BOP), periodontal pocket depth (PPD), visible plaque index (VPI), aMMP-8 and TREM-1.

Visible plaque index percentage (VPI%) was significantly higher in those with a positive aMMP-8 test ($p = 0.005$). Bleeding on probing percentage (BOP%) almost reached a statistically significant difference ($p = 0.052$). Periodontal treatment intervention decreased aMMP-8 levels assessed by a change in test stick result from positive (+) to negative (-) during the monitoring period.

In conclusion, genetic polymorphisms of *MMP3* and *VDR* genes are linked to subclinical periodontitis in Finnish adolescents. Only tentative genetic aetiology for dental caries could be observed. Elevated PGLYRP1 levels in adolescents with gingivitis and subclinical periodontitis and its positive correlation with TREM-1 and aMMP-8 may indicate an association of PGLYRP1 with early stages of periodontal disease. However, it has a lower discriminating capacity and is therefore a less reliable marker alone in the diagnosis of subclinical periodontitis in adolescents. The aMMP-8 chairside mouth rinse test identified and alerted adolescents with poor oral hygiene at risk for subclinical periodontitis without detectable and visible manifestations of the illness. Young patients at risk must be identified to enable early preventive interventions and treatment.

## 1. INTRODUCTION

Dental caries is the most common infection (Shungin et al. 2019) and periodontitis is the sixth most common chronic inflammatory infectious disease in the world (Boelen et al. 2019). Even adolescents suffer from an early stage of periodontitis, subclinical periodontitis (Heikkinen et al. 2008; Heikkinen 2011). In Finland, 75% of adults have periodontal disease and 79% have manifest caries lesions or fillings on the teeth (Suominen et al. 2018). The prevalence of enamel caries lesions is high (99%) among adults in Northern Finland (Laajala et al. 2019). Several genes are likely to have an influence on dental caries, but the role of genetic factors remains obscure, while the genetic polymorphism of periodontitis has been more studied and established (Shungin et al. 2019).

Dental caries is a multifactorial disease caused by environmental and behavioural risk factors. Demographic parameters, including age, sex, and ethnicity, are important predictors for caries, as are environmental factors such as tooth-brushing behaviours, water fluoride levels and family education level (Wang et al. 2012).

Periodontal diseases are diagnosed mainly by clinical examination, including measurement of visible plaque, bleeding on probing, root calculus, deepened periodontal pocket depths, and alveolar bone loss, complemented with radiological examination when necessary. These measurements indicate only the past experience, not the current situation, and do not predict the future. They have low sensitivity and low positive predictive value (Kinane et al. 2017). Periodontitis has few symptoms, especially at onset of the disease, and progresses slowly (Laine et al. 2012). In subclinical periodontitis, mainly gingival bleeding and shallow periodontal pockets exist. Therefore, it is important to diagnose and predict the disease as early as possible and to estimate how treatment would impact the disease.

Periodontitis is associated with complex interactions between periodontal bacteria, the host's inflammatory response, and genetic, environmental, and behavioural risks. The "red complex" bacteria, *Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia*, are characteristic in subgingival biofilm (Socransky et al. 1998). Bacterial substances, especially lipopolysaccharides, activate lymphocytes to produce cytokines, which manifest proinflammatory activities and play key roles in periodontal tissue breakdown through collagenolytic enzymes such as matrix metalloproteinases (MMPs) (Menezes-Silva et al. 2012).

Saliva is an easily accessible biological fluid and contains a variety of disease-related biomarkers, which makes it a potential diagnostic tool. Analysis of oral fluid accurately reflects periodontal diseases (Rathnayake et al. 2013; Nwhator et al. 2014; Heikkinen et al. 2016a; Sorsa et al. 2016). Peptidoglycan recognition protein 1 (PGLYRP1) and triggering receptor expressed on myeloid cells 1 (TREM-1) are known to be involved in proinflammatory production during infection (Read et al. 2015; Silbereisen et al. 2019). Both PGLYRP1 and TREM-1 levels are increased in periodontitis. MMPs also have an important role in the pathology of periodontitis. MMPs are a group of enzymes that increase significantly in pathological conditions. MMP-8 is a remarkable collagenase in tissue destructive oral disease such as periodontitis (Sorsa et al. 2004, 2006). The active form of MMP-8 (aMMP-8) is a risk factor for periodontitis in both adults (Nwhator et al. 2014; Sorsa et al. 2016) and adolescents (Heikkinen et al. 2016a).

Chairside assay technologies have been developed some years ago for the rapid detection of pathologically elevated levels of MMP-8 (Sorsa et al. 2016). The aMMP-8 chairside test is easy to use (Leppilahti et al. 2014; Heikkinen et al. 2016a; Sorsa et al. 2016). The result is provided in 5 minutes and graded by eye as positive or negative. The test may identify active periods of periodontitis, and therefore, has great value in reporting ongoing periodontal breakdown (Sorsa et al. 2020).

More attention must be paid to early diagnostics, prevention, and treatment of periodontitis because it can lead to loss of teeth, impacting also general health, and its treatment by specialists is expensive. Its detection could prevent the irreversible changes of the disease, thereby saving money for individuals and nationally. Young risk patients must be recognized early enough, long before any signs of the disease for preventive intervention. Treatments should focus on the early stage of the disease.

## 2. REVIEW OF THE LITERATURE

### 2.1. Periodontal diseases

#### 2.1.1 Classification of periodontal diseases

According to a new classification of periodontal diseases, both forms of periodontitis (chronic and aggressive) are now grouped under the single category periodontitis and are moreover defined as to staging and grading (Papapanou et al. 2018). Staging is related to the severity and complexity of disease, whereas grading concentrates on biological features of the disease, periodontitis progression, and risk assessment.

The four stages are based on 1) severity, e.g. interdental clinical attachment loss, radiographic bone loss, and possible tooth loss, 2) complexity based on probing depth and horizontal or vertical bone loss, and 3) extent and distribution described as localized, generalized, or molar/incisor pattern. Grades A, B, and C are assigned according to primary criteria (direct or indirect progression) and grade modifiers. Direct progression is measured by radiographic bone loss or clinical attachment loss and indirect progression by percentage bone loss per age or biofilm phenotype. Grade A describes slow, grade B moderate, and grade C rapid rate of progression. Grade modifiers are based on the risk factors smoking and diabetes. (Papapanou et al. 2018).

#### 2.1.2 Gingivitis

Gingivitis is an inflammatory state of the gingiva, the soft tissues surrounding the teeth (Figure 1). Gingivitis always precedes periodontitis. However, gingivitis does not always progress to periodontitis (Brown & Löe 1993). Gingivitis affects 50-90% of the adult population worldwide (Pihlström et al. 2005). Microorganisms quickly colonize tooth surfaces after neglection of oral hygiene procedures. Within a few days, clinical signs of gingivitis appear. Gingivitis is a direct immune response to dental microbial plaque and is modified by several factors such as smoking, certain drugs, and hormonal changes (Kinane 2001). Clinical signs of gingival inflammation include changes in colour and texture. These inflammatory changes can be reversed when adequate tooth cleaning methods are resumed (Löe et al. 1965). Bleeding on probing (BOP) has been widely used as a sign of gingival inflammation and to determine the periodontal health of a patient (Lang et al. 1986).

### 2.1.3 Periodontitis and subclinical periodontitis

**Periodontitis**

Periodontitis is an infectious inflammatory disease destroying the tooth-supporting tissues, including the periodontal ligament, bone, and soft tissues (Figure 1), and if untreated, leads to tooth loss (Kinane 2001; Ramseier et al. 2017). Periodontitis is a multifactorial disease in which genetic and environmental factors affect the clinical outcomes (Page et al. 1976). Changes in periodontium are often severe when the diagnosis is made. One of the major determinants of periodontal diseases is pathogenic bacterial biofilm, dental plaque that activates an immune response (Sudhakara et al. 2018). Periodontitis is very common in all populations. Some 5-20% of adults suffer from severe generalized periodontitis depending on how the disease is defined and the age of the person (Burt 2005; Kassebaum et al. 2014).

**Subclinical periodontitis**

Subclinical periodontitis may correspond to stage I periodontitis, according to the new classification of periodontal diseases (Caton et al. 2018). Stage I signifies maximum probing depth ≤ 4 mm (Papapanou et al. 2018). In its early phase, periodontitis has no remarkable visible signs in clinical or radiological examination (Heikkinen et al. 2017). Subclinical periodontitis is situated between b and c and is emphasized towards c in Figure 1. The main risk factor for subclinical periodontitis in adolescents may be partly calcified, dysbiotic bacterial biofilm (Heikkinen et al. 2019). Periodontitis disease occurs also in adolescents; up to 10-15% of 15- to 16-year-olds suffer from the early stage of periodontitis, subclinical periodontitis (Heikkinen et al. 2008).

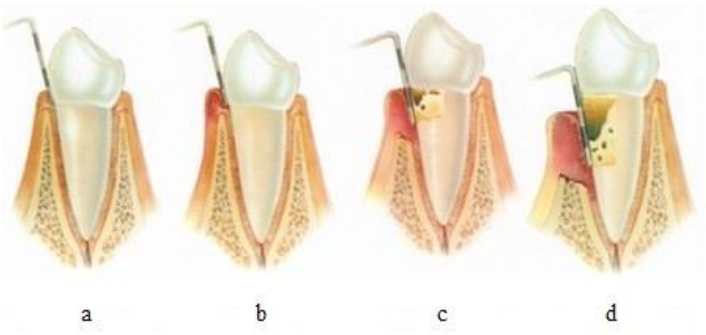


**Figure 1**. State of periodontium.
a health periodontium, b gingivitis, c and d periodontitis. *Figure with permission from Henrik Kiige, Sakala Hambaravi.*

### 2.1.4 Pathogenesis

Periodontitis is a biofilm-induced chronic inflammatory disease (Hajishengallis 2014). The 'red complex' species, *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), and *Treponema denticola* (*T. denticola*), is associated with chronic periodontitis, whereas *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) is found in aggressive periodontitis (Socransky et al. 1998). These pathogens are members of the normal microbiota, but do not cause disease because of their low levels. Changes in ecological conditions, such as dysbiosis in microbial composition, increase the number of pathogens and lead to periodontitis (Marsh et al. 2003; Hajishengallis 2014).

*P. gingivalis,* the Gram-negative anaerobe bacterium, is one of the major pathogens implicated in inflammatory responses in periodontitis. It induces the expression of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)-α, stimulating the immune response during infection (Bostanci & Belibasakis 2012). *T. forsythia* interacts with numerous other bacteria, but does not play a crucial role in regulating the bacterium's growth in a multispecies biofilm (Bloch et al. 2017). *T. denticola* is a spirochete and may comprise more than 30% of the periodontal subgingival microflora (Kinane et al. 2001), but it is a very minor component in healthy periodontium (Hajishengallis 2014). *A. actinomycetemcomitans* is an aerobic Gram-negative bacteria that is regarded as a key micro-organism in the early onset of periodontitis (Cappelli et al. 1994; Hall et al. 1991; Kinane et al. 2001).

Periodontal tissue homeostasis depends on the host and the periodontal microbiota as well as on immune defences against microbial attacks. However, recently, it has been suggested that the pathogenesis of periodontitis implicates polymicrobial synergy and dysbiosis, called the PSD model, as opposed to the traditional model of infectious disease caused by select periopathogenes such as the 'red complex' (Hajishengallis & Lamont 2012). This model consists of both Gram-negative and Gram-positive bacteria. The transition to periodontitis requires both a dysbiotic microbiota and a susceptible host (Darveau 2010; Hajishengallis 2014). Keystone pathogens, even at low amounts, elevate community virulence, leading to dysbiotic circumstances and paralysing the immune system because of overall inflammatory response (Lamont & Hajishengallis 2015). Inflammation and dysbiosis strengthen each other.

Biofilm is required, but not in itself sufficient to induce periodontitis (Hajishengallis 2014). Dental plaque accumulation causes gingivitis, which does not necessarily lead to periodontitis, suggesting

that stable gingivitis represents a protective host response (Graves et al. 2011). Thus, some individuals remain free of periodontitis despite massive dental plaque accumulation (Laine et al. 2012); however, poor oral hygiene is related to periodontitis (Lertpimonchai et al. 2017).

Some local and systemic risk factors have an effect on how plaque accumulation leads to gingival inflammation and its progression. Local factors for gingivitis, such as retention factor, encourage plaque accumulation by either inhibiting its removal and/or creating circumstances for plaque increase (Chapple et al. 2018). Systemic risk factors – smoking, pharmacological agents, and sex steroid hormones – influence negatively the immune inflammatory response (Chapple et al. 2018). Robust evidence shows an association of periodontal diseases with systemic diseases such as cardiovascular disease and diabetes (Nazir et al. 2017).

### 2.1.5 Brief review of genetic background related to periodontitis: *MMP3* and *VDR*

Elevated levels of MMPs, such as MMP-3, have been detected in gingival crevicular fluid, peri-implant sulcular fluid, and gingival tissue of periodontitis patients (Li et al. 2016). Polymorphism in the gene encoding MMP-3 has been shown to be associated with chronic periodontitis (Astolfi et al. 2006; Letra et al. 2012; Li et al. 2012; Weng et al. 2016), however Itagaki et al. (2004) did not support this hypothesis. The strongest associations were seen with two *MMP3* SNPs (rs679620 and rs650108) (Letra et al. 2012). Polymorphism in the *MMP3* (rs679620, rs520540, rs639752) encoding gene was also found by Heikkinen et al. (2016b).

Vitamin D, especially the active form of vitamin $D_3$ 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2 D_3$] plays an important role in bone homeostasis and immunity (Amano et al. 2009). Patients with chronic periodontitis have been reported to have low levels of serum vitamin D (Bhargava et al. 2019; Bonnet et al. 2019). Recently Bhargava et al. (2019) observed a statistically significant relationship between serum 25(OH) D level and periodontal parameters such as gingival index, probing pocket depth, and clinical attachment level.

Vitamin D activates monocytes, stimulates cell-mediated immunity, and suppresses lymphocyte proliferation, and thus, may be interpreted to indicate a close relationship between *vitamin D receptor* (*VDR)* gene polymorphism and the immunological action (Tachi et al. 2003). *VDR* gene polymorphism (tt genotype) might be a risk factor for chronic periodontitis. Tachi et al. (2003) reported *Taq I* polymorphism in the *VDR* gene to be associated significantly with chronic periodontitis among Japanese subjects, whereas Wang et al. (2009) reported a similar association in

Chinese patients. Wan et al. (2019) showed a correlation between *VDR Fok*I gene polymorphisms and aggressive periodontitis and between *Taq*I polymorphism and periodontitis in the Caucasian population. *VDR Apal*, however, was not correlated with periodontitis. Polymorphism of *VDR* (rs2228570) seems to be linked to the early stage of periodontitis (Heikkinen et al. 2016b). There are also contradictory results. Nazemisalman et al. (2019) found no statistically significant association between *VDR* gene polymorphism and periodontitis in an Iranian population, nor did Gunes et al. (2008) in Turkish patients.

### 2.1.6 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a group of genetically distinct, but structurally related proteolytic enzymes. The expression and activity of MMPs in non-inflamed tissue is low but, increase significantly in various pathological inflammatory and malignant conditions (Nagase & Woessner 1999; Visse & Nagase 2003; Sorsa et al. 2006). MMPs play a key role in the tissue destruction characteristic of chronic periodontitis. The main function of MMPs is removal of extracellular matrix (ECM) during tissue resorption and progression (Nagase & Woessner 1999). Especially MMP-8 (collagenase-2) is a major collagenolytic protease in periodontitis (Sorsa et al. 2004, 2006). Other collagenases, MMP-1 (collagenase-1) and MMP-13 (collagenase-3), seems to play minor role in chronic periodontitis (Gonçalves et al. 2013). Since the major structural protein of the periodontium is collagen, the assessment of MMP levels during inflammation will reflect the association of MMPs with collagen destruction in periodontitis (Kinane et al. 2001). MMPs have also anti-inflammatory effects in defence of the host by processing anti-inflammatory cytokines and chemokines and regulating immune responses (Sorsa et al. 2006). Besides MMPs being a component of bone destruction (Jin et al. 2014), other proteinases, such as cathepsin-K, may also be involved in osteoclastic bone destruction in periodontal disease (Mogi & Otogoto 2007).

MMPs are zinc-containing metalloendopeptidases. Each MMP consists of a specific sequence of domains. The domain structure includes the propeptide domain, the catalytic domain, and the hemopexin domain. Some MMPs have additional domains, such as a transmembrane or a cytoplasmic domain (Nagase & Woessner 1999; Visse & Nagase 2003).

Twenty four members of the MMP family have been identified. Of these, 23 have been found in the human genome. MMPs are classified into the following five categories (Visse & Nagase 2003; Sorsa et al. 2006):

Collagenases: MMP-1, MMP-8, MMP-13, and MMP-18
Gelatinases: MMP-2 and MMP-9
Stromelysins: MMP-3, MMP-10, and MMP-11
Matrilysins: MMP-7 and MMP-26
Membrane-type MMPs: MMP-14, MMP-15, MMP-16, MMP-24, MMP-17, and MMP-25
Seven MMPs are not classified in the above categories: MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, and MMP-28

#### 2.1.6.1 MMP activation

All MMPs are synthesized as prepro-enzymes and are secreted as inactive proMMPs (Nagase & Woessner 1999). MMPs are usually activated extracellularly or at the cell surface. MMPs may also be activated intracellularly (Visse & Nagase 2003; Sorsa et al. 2004). The mechanism regulating MMP activation varies depending on the specific tissue and the disease microenvironment (Franco et al. 2017). MMP activity is regulated through gene expression, proenzyme activation, and enzyme inhibition (Visse & Nagase 2003; Sorsa et al. 2004).

Proteolytic activation of MMPs is stepwise in many cases (Nagase 1997). Activated MMPs are able to activate each other. As an example, MMP proteolytic activation is described by Franco et al. (2017) as follows: MMP-14 activates proMMP-13; MMP-13 activates proMMP-9, which in turn activates proMMP-2 and proMMP-13.

MMP activation is also possible through reactive oxygen species (ROS), an oxidative non-proteolytic route, through direct enzyme oxidation. Oxidant hypochlorous acid (HOCl), catalysed by myeloperoxidase (MPO), can activate proMMP-8 and proMMP-9 (Saari et al. 1990). MPO may also activate directly proMMP-8 and proMMP-9. These findings suggest that MPO might play an important role in activating MMP-8 during periodontal disease progression (Franco et al. 2017). Interestingly, MPO is found in primary granules from neutrophils, like MMP-8 and MMP-9 (Kowolik & Grant 1983). HOCl and hydrogen peroxide ($H_2O_2$) can also modify the protease and anti-protease balance, activating latent MMPs and inactivating the tissue inhibitor of MMPs (TIMPs) (Saari et al. 1990; Sorsa et al. 2004).

Activation or activity of MMPs are inhibited by TIMPs (Uitto et al. 2003; Visse & Nagase 2003; Sorsa et al. 2006). TIMP family consists of four members (TIMP 1-4).

Additionally, MMPs can be activated by host inflammatory mediators like TNF-α, IL-1β, and microbial and host-derived proteases (Sorsa et al. 1992) and in vitro by chemical agents (Visse & Nagase 2003). Serine proteinase plasmin can activate proMMPs (Visse & Nagase 2003).

#### 2.1.6.2 Matrix metalloproteinase-8

As mentioned, MMP-8 is the major collagenase in periodontitis (Sorsa et al. 2004). It is secreted mainly by neutrophil leukocytes, and thus, is known also as neutrophil collagenase. MMP-8 is also produced by epithelial cells, fibroblasts, endothelial cells, monocytes, macrophages and plasma cells (Sorsa et al. 2004).

MMP-8 is released by polymorphonuclear leukocytes (PMNs) in an inactive form, but is almost completely in active form during the progressive state of periodontitis (Sorsa et al. 1988). The active form, aMMP-8 has been shown to be a risk factor for periodontitis among adults (Nhwator et al. 2014; Sorsa et al. 2016) and adolescents (Heikkinen et al. 2008, 2010, 2016a, 2016b, 2017, 2019). MMP activation in general is described in Section 2.1.6.1. MMP-8 can also be activated during inflammation by bacteria, e.g. *P. gingivalis* and *T. denticola.* Bacterial surface molecules, such as lipopolysaccharides (LPSs), stimulate host epithelial cells to release proinflammatory mediators and cytokines such as TNF-α and IL-1β and proteases (Sorsa et al. 1992). MMP-8 is also activated by other MMPs. Treatment of periodontitis decreases the aMMP-8 levels in oral fluids (Mäntylä et al. 2003; Heikkinen et al. 2010; Sorsa et al. 2016).

### 2.1.7 Potential other biomarkers involved in periodontitis: PGLYRP1, TREM-1, and PMN elastase

#### PGLYRP1

Peptidoglycan recognition proteins (PGLYRPs) are innate immunity proteins that not only defend the host against infections and control inflammation but also regulate and maintain normal microflora (microbiome) (Royet et al. 2011). PGLYRP1 is one out of four members of PGLYRPs in mammals. PGLYRP1 was initially presented as a soluble protein in granules of PMNs. These proteins kill Gram-positive and Gram-negative bacteria by interacting with the cell wall peptidoglycan (Dziarski & Gupta 2006). PGLYRP1-knockout mice models demonstrated a reduced antibacterial activity and a higher susceptibility to bacterial infections than in wildtype mice due to impaired PMN functions with regard to intracellular killing and bacterial digestion (Dziarski et al. 2003). Follow-up studies applying a PGLYRP1-deficiency model of induced colitis showed that PGLYRP1 protects mice from developing inflammation by downregulating the interferon (IFN)-γ inflammatory response (Saha et

al. 2010). In contrast to the rather protective role of PGLYRP1 in colitis, PGLYRP1 was shown to enhance asthma, arthritis, and skin inflammation, i.e. dermatitis and psoriasis (Park et al. 2013). PGLYRP1´s inflammation propagating properties in bacterial infection are attributed to its receptor, as it was recently discovered as a functional ligand for the triggering receptor expressed on myeloid cells 1 (TREM-1) (Read et al. 2015). Other PGLYRPs (PGLYRP 2-4) are expressed in epithelial cells of many organs, including salivary glands, throat, tongue, esophagus, stomach, intestine, and skin (Lu et al. 2006; Liu et al. 2001).

**TREM-1**

Triggering receptor expressed on myeloid cells 1 (TREM-1) is a member of the immunoglobulin superfamily. It is a transmembrane receptor expressed on neutrophils and monocytes and is released during infections, especially bacterial infections (Gibot & Cravoisy 2004; Forrester et al. 2017). TREM-1 has the capacity to amplify proinflammatory cytokine production. MMP-8 is responsible for the proteolytic cleavage of mature, membrane-bound TREM-1 (Bostanci & Belibasakis 2012; Weiss et al. 2017). MMPs can process membrane-bound TREM-1 to its soluble form sTREM-1 (Bostanci & Belibasakis 2012; Weiss et al. 2017). Bacterial infection also can upregulate sTREM-1, which in turn strengthens inflammation (Bouchon et al. 2001). Soluble TREM-1 is shed into human biological fluids during the course of infection (Gibot & Cravoisy 2004; Forrester et al. 2017). Various studies in adults showed significantly higher levels of TREM-1 in serum, saliva, and gingival crevicular fluid (GCF) in patients with periodontitis than in healthy individuals (Bostanci et al. 2013a; Belibasakis et al. 2014; Willi et al. 2014). TREM-1 is involved in alveolar bone resorption during the process of periodontitis in mice (Bostanci et al. 2019).

Periodontal pathogen *P. gingivalis* was shown to stimulate TREM-1 production in monocytes, promoting a shift from cell-membrane form to sTREM-1, which also increases pro-inflammatory cytokine production, including TNF-α and IL-1β (Bostanci et al. 2011, 2013b). TREM-1 and PGLYRP1 were reported to have a positive correlation with MMP-8 in saliva of adult periodontitis patients with chronic kidney disease (Nylund et al. 2018). On the other hand, blockage of MMPs by low-dose tetracycline (main action due to MMP-8 inhibition) reduced TREM-1 release by human monocytes and neutrophils into the extracellular milieu, and thus, may partly explain the presence of TREM-1 in saliva (Bostanci & Belibasakis 2012). The increase in MMP-8 levels in saliva in response to biofilm accumulation resembled TREM-1 release into saliva (Silbereisen et al. 2019).

**PMN elastase**

Neutrophil elastase (NE) activity is increased in many diseases (Geraghty et al. 2007). NE, stored in granules of PMN, is one of the most destructive enzymes and is rapidly released when activated (Sorsa et al. 2006; Geraghty et al. 2007). PMNs play an important role in the development of inflammation such as periodontitis. PMN leukocytes are important cells because they form the first line defence against pathogens (Kinane et al. 2001). PMN elastase also activates MMP-8. Therefore, this increase in PMN elastase levels in periodontitis may also cause TREM-1 shedding (Bostanci et al. 2013b). However, smoking decreased PMN elastase levels in adolescents (Heikkinen et al. 2010).

### 2.1.8 Cytokines

Cytokines control inflammatory and immune responses. According to Kinane et al. (2001), the cytokines can be split into two groups those involved in inflammatory and immune reactions (interleukins, chemokines, and interferons) and those involved in tissue growth and repair.

TNF-α is a multipotential cytokine produced mainly by macrophages. It increases the number of PMN leukocytes and monocytes to the sites of inflammation and causes tissue destruction and bone loss (Birkedal-Hansen 1993). IL-6 is produced by macrophages, fibroblasts, lymphocytes, and endothelial cells, while IL-8 is produced by PMN leukocytes, monocytes, fibroblasts, and keratinocytes. IL-6 may be the cytokine through oestrogen and progesterone to exert its effects on gingiva, whereas IL-8 plays an important role in the accumulation of leukocytes at the sites of inflammation (Kinane et al. 2001).

## 2.2 Periodontal examination and diagnostics

### 2.2.1. Examination of periodontium

Bleeding on probing (BOP) is measured with a blunt periodontal probe which is inserted to the bottom of the gingival pocket and moved gently with a strength of 20g (Ainamo & Ainamo 1994) along the tooth surface. If bleeding is achieved from the pockets, the site examined is considered inflamed (Lindhe 1985). Bleeding is registered after 60 seconds for all teeth at four or six sites (Ainamo & Bay 1975). Bleeding sites are recorded as bleeding site percentage of all sites (BOP%). BOP values below 20% have significantly lower risk for disease progression (Joss et al. 1994). According to the new classification of periodontal diseases, the value is even lower, set on 10% (Chapple et al. 2018).

Visible plaque index (VPI) and root calculus (RC) are examined from WHO index teeth (DD 16, 11, 24, 36, 41, 44) and registered for all teeth and four or six sites (Ainamo & Bay 1975).

The pocket depth, i.e. the distance from the gingival margin to the bottom of the dental pocket, is measured with the same blunt periodontal probe as with bleeding on probing. The pocket depth is assessed at each surface of all teeth. Pocket depth ≥ 4 mm is recorded as the periodontal status. Pockets < 4 mm are regarded to lie within normal variation (Ainamo & Bay 1975).

Clinical attachment loss (CAL) is diagnosed by measuring the pocket depth together with radiological findings. In the radiograph, disease of the periodontium is recognized by reduced height of the alveolar bone (Nieminen et al. 1993). Attachment loss is considered normal at values below 2mm (Nieminen et al. 1993) measured from the cementoenamel junction to the marginal alveolar bone (Armitage 2004).

All of the above-mentioned measures concern past tissue destruction, destruction that has already happened. Information about the current stage of the periodontium is not available.

### 2.2.2 Oral fluids

Oral fluids include saliva, mouth rinse, and GCF. Oral fluids are useful in diagnosing oral diseases. Analyses of oral fluid mirror well the progress of periodontal diseases (Rathnayake et al. 2013; Nwhator et al. 2014; Heikkinen et al. 2016a; Sorsa et al. 2016).

Whole saliva is a mixture of oral fluids; it contains secretions of the minor and major salivary glands as well as GCF, excreted bronchial secretions, serum and blood cells from oral wounds, micro-organisms, host tissue and cell degradation products, and food debris (Kaufman & Lamster 2000; Sorsa et al. 2004). It is possible to detect biomarkers for oral diseases within saliva samples (Rathnayake et al. 2013).

Mouth rinse reminds saliva samples (Pauletto et al. 2000). In the mouth rinse sampling, saliva is washed out of the oral cavity by rinsing the mouth with tap water before actual sampling. The actual sample is collected after washout by rinsing the mouth again (Romanelli et al. 1999; Mancini et al. 1999).

GCF is most frequently collected by insertion of filter paper strips to the bottom of the gingival sulcus for 30 seconds (Mäntylä et al. 2003). Obtaining pure GCF, especially from posterior sites, is especially difficult because of easy contamination by saliva and plaque (Smith & Camp 1993).

Peri-implant sulcular fluid (PISF) is also collected by inserting paper strips below the mucosal margin of the peri-implant sulcus (Alassiri et al. 2018).

### 2.2.3. Active matrix metalloproteinase (aMMP)-8 chairside test

The aMMP-8 chairside test (PerioSafe®, Dentognostics GmbH, Jena, Germany) has proven to be useful in diagnostics, screening, predicting, planning treatment, and follow-up of periodontal diseases (Nwhator et al. 2014; Heikkinen et al. 2016a; Sorsa et al. 2016; Alassiri et al. 2018; Leppilahti et al. 2018; Räisänen et al. 2018, 2019). This chairside point-of-care (PoC) lateral-flow immunotest is practical, sensitive, specific, inexpensive, non-invasive, and rapid, and it never causes bacteraemia, differing from bleeding on probing. The cut-off point was 20 ng/ml (Nwhator et al. 2014; Heikkinen et al. 2016a; Alassiri et al. 2018). The test functions also quantitatively (Nwhator et al. 2014; Heikkinen et al. 2016a; Sorsa et al. 2017; Alassiri et al. 2018). The test can differentiate healthy and gingivitis sites from periodontitis sites (Mäntylä et al. 2003; Heikkinen et al. 2016a).

The result can also be read by quantitative analysis by the enzyme-linked immunosorbent assay (ELISA) and the time-resolved immunofluorometric assay (IFMA) (Sorsa et al. 2017; Alassiri et al. 2018). The aMMP-8 chair side test, IFMA, and dentoELISA use the same monoclonal antibodies (Sorsa et al. 2010). IFMA and dentoELISA correlate and chair side test results are also in line with them (Sorsa et al. 2010) in detecting elevated aMMP-8 concentrations in periodontitis, affected GCF and mouth rinse samples.

The test has a diagnostic sensitivity and specificity of 76-90% and 96%, respectively (Nwhator et al. 2014; Heikkinen et al. 2016a; Sorsa et al. 2017; Alassiri et al. 2018). The sensitivity of a test describes how well the test can find patients with the disease, whereas the specificity of a test describes how well the test can find patients without the disease.

## 2.3 Dental caries

Acidogenic bacteria cause damage to tooth enamel in the presence of fermentable carbohydrates. When the pH at the surface of the tooth falls below 5.5, demineralization proceeds and without eradication of the biofilm leads to tooth decay (Featherstone 2000). Demineralization and destruction of enamel are followed by destruction of the collagenous organic matrix of dentin (Tjäderhane et al. 1998).

Like most developed Western countries, the prevalence of dental caries among children in Finland has decreased from 1970 to 1990 (Petersson & Bratthall 1996). During the decline of the caries rate its occurrence has been polarized (Seppä 2000), and it remains the most common chronic childhood disease (Wang et al. 2012). In 1993, 26% of 15-year-olds in Finland were caries-free, and 55% of all caries occurred in 10% of the subjects (Vehkalahti 1997). From data collected after 2000, the number of decayed, missed and filled permanent teeth (DMFT) was among 12-year-olds 1.0 ± 0.2 in Western Europe. The corresponding score in Finland was 0.9 in 2018 (National Institute for Health and Welfare, Sotkanet, Finland 2018).

Even though the decline is considerable, with a 90% reduction in DMFT for 12-year-olds in Western Europe and the USA, caries still affects 60-90% of children globally and remains the most widely spread non-communicable disease (Lagerweij & van Loveren 2015). In Finland, caries prevalence among children and adolescents has shown a slight tendency for an increase (Suni et al. 2008), although evidence-based information is missing.

Due to the multifactorial nature of caries disease, demographic parameters, including age, sex, and ethnicity, are important predictors for caries (Wang et al. 2012). Other environmental and favourable socio-economic factors, such as frequent tooth-brushing behaviours, sufficient water fluoride levels, and high family education level are protective factors for primary dentition (Levy et al. 2003). However, reduced salivary flow, hypomineralized tooth structure, and unfavorable dietary habits among others, enhance the risk for caries development and progression. Indications of genetic susceptibility to dental caries have emerged (Shungin et al. 2019). However, spare information exits about the effects of genetic background on caries in adolescents (Wang et al. 2010).

### 2.3.1 Pathogenesis

*Streptococcus mutans* (*S. mutans*) is the primary agent of dental caries. However, in a Swedish population, caries-active adolescents were mainly colonized by *Actinomyces*, *Selenomonas*, *Prevotella* and *Capnocytophaga* species (Johansson et al. 2016). These findings suggest that *S. mutans* as a primary agent of dental caries is less pronounced in populations with preventive strategies. *S. mutans* and *Streptococcus sobrinus* are prevalent in populations without routine caries treatment or preventive programmes. In addition to producing acid through their metabolism, *S. mutans* participate in biofilm formation by producing extracellular polysaccharides, matrix of biofilm, followed by cell–cell aggregation, sucrose-dependent stabilization, and eventual biofilm maturation (Li & Wang 2014).

Oral lactobacilli are members of a group of bacteria implicated in caries progression. Oral lactobacilli together with *S. mutans* were significantly associated with the highest dental caries indices in children with primary dentition. *Lactobacillus fermentum* was the most prevalent, and its presence was related to high caries indices (Lapirattanakul et al. 2020).

A new ecological aspect has recently elucidated, namely bacterial acid-induced adaptation and selection in microbiota. A major ecological driver has traditionally been bacteria-induced acid formation in which a proteolytic stage has been added. MMPs and cysteine cathepsins are both participants in activated forms of the caries process with the difference that MMPs start to function in neutral pH and cathepsins under acidic circumstances (Takahashi & Nyvad 2016).

### 2.3.2 Brief review of genetic background related to dental caries: *MPO, DDX39B, VDR, LTA,* and *MMP3*

Myeloperoxidase (MPO) is a defensive peroxidase enzyme that is encoded by the *MPO* gene. It is most abundantly expressed in neutrophil granulocytes in saliva and is a strong biomarker for both acute and chronic inflammatory conditions. Its main role is to produce hypochlorous acid to carry out antimicrobial activity (Kinkade et al. 1983). The association of *MPO* gene with dental caries or even oral diseases is discussed little in the literature. *MPO* gene could increase or decrease the risk of fluorosis among individuals in endemic areas (Pramanik & Saha 2017). A significant association between *MPO* polymorphism and oral lichen risk has been found (Wu et al. 2015). *MPO* gene polymorphism has been shown to be associated with coronary artery disease and lung cancer (Gu et al. 2014; Li et al. 2017).

DDX39B (BAT1) belongs to the DEAD-box family of ribonucleic acid (RNA)-binding proteins and is encoded in the central human major histocompatibility complex (MHC). The region contains numerous genes involved in immune and inflammatory responses. BAT1 is a negative regulator of inflammation (Allcock et al. 2001). *DDX39B* encodes the RNA helicase known to regulate expression of TNF-α and IL-6 (Mendonça et al. 2014). Both of these cytokines are well known in inflammatory conditions. BAT1 protein may be directly responsible for the genetic association and can down-regulate inflammatory cytokines (Price et al. 2004).

Vitamin D regulates the calcium metabolism of the human body and maintains sufficient levels of calcium and phosphate in the serum for the mineralization of bones. Vitamin D takes part in the

formation of teeth, especially in the calcification of enamel and dentin (Hujoel 2013), and also has an important role in the immune system (Clark et al. 2016). Nutrition disorders lead to uncontrolled changes in the immune system, preventing the immune response to oral infections such as caries and periodontitis (Willershausen et al. 2011). The function of Vitamin D is modulated by vitamin D receptor (VDR) protein (Sutton & MacDonald 2003). The activity of VDR protein is affected by *VDR* gene polymorphisms (Valdivielso & Fernandez 2006). Most of the *VDR* gene polymorphisms include variants in *Bsm I*, *Apa I, Taq I* and *Fokl* genes. These polymorphisms have associations with different inflammatory and autoimmune diseases (Székely & Pataki 2012; Wöbke et al. 2014).

Lymphotoxin (LT)-α or TNF-β is a protein that in humans is encoded by the *LTA* gene, which is located on chromosome 6 and is in close proximity to the gene encoding the major histocompatibility complex (Nedwin et al. 1985). As a cytotoxic protein, LT-α performs a variety of important roles in immune regulation (Nedwin et al. 1985) and is produced by lymphocytes. Genetic variations of *LTA* gene are associated with susceptibility to myocardial infarction, non-Hodgkin's lymphoma and psoriatic arthritis (Castillo Pedraza et al. 2019).

MMPs have been shown to play important roles in dentin formation and caries progression (Niu et al. 2011). MMP-3 is present in dentin and may take part in dentin matrix formation and its mineralization (Mazzoni et al. 2011). MMP-3 is involved in the breakdown of ECM in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, and in disease processes (Taylor et al. 2008). Menezes-Silva et al. (2012) observed a significant association between MMP-2 and MMP-3 and the presence of large periapical lesions. This suggests that MMPs may be associated with periapical lesion formation caused by untreated deep carious lesions. *MMP-3* gene is likely to be involved in caries (Menezes-Silva et al. 2012; Karayasheva et al. 2016).

### 2.4 Oral health behaviours of adolescents in association with oral diseases

Gingivitis and periodontitis as well as dental caries are caused by poor oral behaviours, such as poor dietary and oral hygiene habits, and smoking, alcohol consumption, and use of snuff (Mäntylä et al. 2006; Heikkinen et al. 2008, 2010, 2019; Johanssen et al. 2014; Kumar et al. 2016; Leite et al. 2018). Food consumption patterns during adolescence are linked to adulthood choices, and poor habits in adolescence pose a risk for developing diseases in adulthood (Diethelm et al. 2012).

In Finland, around 40% of daily energy intake among adolescent is derived from snacks, such as sweets or chocolates, bread, cereal bars, fruit and sugary soft drinks, according to a study on the eating habits of seventh and eighth grades (Hoppu et al. 2008). Daily snacking was associated with restorative treatment need among young Finnish men (mean age 19.6 years) and was also associated with other harmful oral and general health habits (Tanner et al. 2020). According to Hoppu et al. (2008), boys consume more soft drinks, while girls consume more sweets. Over half of the participants ate candies one to two times a week. Of girls and boys, 40% and 28%, respectively, consumed fresh vegetables and 32% and 23% consumed fruits daily. The same result was found in other studies. Boys consumed more soft drinks, whereas girls enjoyed more water, fruit juice, and sweets according to the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) cross-sectional survey (Rey-Lopez et al. 2011). Adolescents in Europe consume far too little fruit, vegetables, and milk products and far too much meat, meat products, oils, fats, and sweets (Diethelm et al. 2012). In Spain, girls ate healthier than boys, except at breakfast (Costa-Tutusaus & Guerra-Balic 2016).

Tooth brushing is the most common recommendation for oral hygiene (Tonetti et al. 2015), and it is generally known that brushing with fluoride toothpaste prevents dental caries (Vanobbergen et al. 2001; Kumar et al. 2016). The National Institute for Health and Welfare in Finland carries out a School Health Survey for adolescents in eight and ninth grades (aged 14-16 years) every two years (National Institute for Health and Welfare 2019) (Table 1). The survey has revealed that nearly two-thirds (70.5%) of girls and half (47.6%) of boys brushed their teeth more than once a day, but tooth brushing score overall decreased for girls and increased for boys when comparing results between the years 2017 and 2019 (Table 1). In Spain, nearly two-thirds of adolescents brushed their teeth more than once per day, and the tooth brushing score was higher for girls (Costa-Tutusaus & Guerra-Balic 2016).

Smoking is widely recognized as a behavioural risk factor for periodontitis (Mäntylä et al. 2006; Johannsen et al. 2014; Leite et al. 2018) and also has an effect on the periodontal health of adolescents (Heikkinen et al. 2008, 2010). Daily smoking has decreased among adolescents in Finland (Table 1). Alcohol consumption was moderate (Table 1), whereas it is common among European adolescents (Samuelson 2000). A strong association between alcohol and tobacco use has been found among elementary and high school students also in Greece (Tsiligianni et al. 2012). In Finland in 2019, more boys (15.6%) than girls (9.8%) had experienced snuff use and the majority had never used drugs (Table 1).

**Table 1**. National Institute for Health and Welfare: School Health Survey for adolescents in eight and ninth grades in 2019 and 2017.

| | **2019** | | **2017** | |
|---|---|---|---|---|
| Health care question | **Girls (%)** | **Boys (%)** | **Girls (%)** | **Boys (%)** |
| Tooth brushing more than once a day | 70.5 | 47.6 | 71.1 | 44.6 |
| Smoking every day | 5.1 | 6.1 | 6.0 | 7.8 |
| Never smoked | 78.0 | 75.3 | 78.4 | 73.4 |
| Snuff use experience | 9.8 | 15.6 | 9.1 | 15.5 |
| Never used alcohol | 61.5 | 60.0 | 63.2 | 60.2 |
| Alcohol use once a week or more often | 3.1 | 5.6 | 3.5 | 6.5 |
| Never used drugs | 93.0 | 89.8 | 94.4 | 90.5 |

### 2.5 Oral health services provided for adolescents in Finland

In Finland, the municipalities are responsible for providing oral health care comprising dental examinations, treatments need, and preventive care. Oral health care is systematic and regular and is free of charge for all citizens aged under 18 years. Thus, virtually everyone under 18 years of age uses public oral health care services.

All students in first, fifth, and eighth grades in Finland are comprehensively invited to an oral examination according to a Government Decree (338/2011) on maternity and child health clinic services, school and student health services and preventive oral health services for children and youths. They also have individually determined examination intervals according to risk assessment. A work division between all dental staff has been developed concerning examinations and preventive care. The Decree has increased the required level of competence for oral health care staff as well as public health nurses. The target in the Decree was to strengthen preventive services to curb both the

need for curative services and the growth of expenditure. Most of the costs of oral health care constitute the costs of salary. The salary of an oral hygienist is about 50% that of a dentist; moreover, the dentist works with a dental nurse. Furthermore, oral hygienists have targeted education in prevention.

### 2.6 Oral health promotion and prevention of periodontitis

Prevention of periodontal diseases consists of the patient's regular good oral hygiene and professional interventions. The most commonly performed preventive measures are the removal of dental biofilm and calcified deposits, professional mechanical biofilm removal, and oral hygiene instructions not to forget risk factor control and to attend recall appointments (Tonetti et al. 2015). Mechanical biofilm removal is currently considered to be an essential procedure for the prevention and treatment of plaque-induced periodontal diseases, emphasizing the importance of good self-care (van der Weijden & Slot 2011).

Prevention of gingivitis is a requirement for the prevention of periodontitis at both the population and the individual level. Furthermore, the risk factors for periodontitis, such as smoking habits and uncontrolled diabetes, must be taken into account in prevention of periodontitis (Tonnetti et al. 2015).

## 3. AIMS OF THE STUDY

Clinical examination with aMMP-8 chairside oral fluid test (Studies I-III and Study IV test group) was performed on Finnish adolescents aged 14-17 years.

The hypotheses of the study were that aMMP-8 levels would be associated with participants at risk for subclinical periodontitis and that treatment and preventive intervention would decrease aMMP-8 levels. We also assumed possible correlations between other proinflammatory biomarkers and subclinical periodontitis. We hypothesized that genetic polymorphism would link subclinical periodontitis and dental caries.

The main aims of the study were as follows:

**Study I**

To determine whether the genetic background of subclinical periodontitis and caries could be detected.

**Study II**

To investigate the genetic background of caries.

**Study III**

To investigate salivary biomarkers, such as levels of PGLYRP1, and its correlation with TREM-1, PMN elastase, and aMMP-8 in adolescents with the goal of identifying at-risk individuals before onset of periodontal disease.

**Study IV**

To evaluate whether participants at risk for subclinical periodontitis could be identified using aMMP-8 chairside mouth rinse test, and further, to determine whether treatment and preventive intervention affects the aMMP-8 test results.

## 4. MATERIALS AND METHODS

### 4.1 Study population I-III

Studies I-III were carried out at the Kotka Health Center in Eastern Finland in 2004-2005 and/or 2014-2015. Participants, aged 15-17 years gave their informed consent for the study and stimulated saliva sampling. Only part of the adolescents consented to DNA analyses. Oral health of all participants was examined. Participants did not have any orthodontic treatment history or mixed dentition. These studies were approved by the Ethics Committees of Kymenlaakso Regional Hospital and Helsinki and Uusimaa Hospital District (Dnro 260/13/03/00/13), Finland.

A flow diagram of Studies I-III is provided in Figure 2.

Study I: The sample was collected in 2014-2015 (Heikkinen et al. 2016a). A total of 47 participants (30 boys and 17 girls) had available saliva and DNA samples. Adolescents was invited to an examination according to their individual examination time. One participant was excluded, because of a discrepant sex check, and thus, the overall number of participants in analyses was 46.

Study II: The sample was collected in 2004-2005 (Heikkinen 2011; Heikkinen et al. 2010) and 2014-2015. Every 15- to 17-year-old living in Kotka was invited to an examination in 2004-2005. Altogether there were 94 participants (47 boys and 47 girls) whose saliva and DNA were collected. One participant was excluded, because of a discrepant sex check, and thus, the overall number of participants in analyses was 93.

Study III: This study was based on data from two cohorts collected in 2004-2005 and 2014-2015. Whole saliva samples (n = 537) were collected from participants (281 boys and 256 girls). One participant was excluded, because of unavailable measurements, and thus the overall number of participants in analyses was 536.

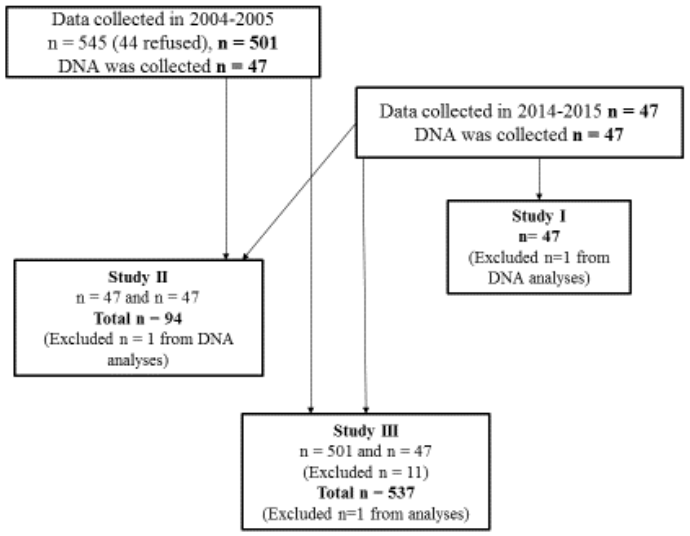


**Figure 2**. Flow diagram of Studies I-III.

### 4.1.1 Clinical examination

Oral health status was recorded according to the World Health Organization (WHO) recommendations (Kramer et al. 1980). Information was collected of decayed, missed, and filled teeth (DMF), decayed teeth (D), initial caries (i), and the highest number and the number of healthy sextants of community periodontal index of treatment need (CPITN). The CPITN consists of values: 0 = healthy, 1 = bleeding gingiva, 2 = calculus, 3 = periodontal pockets ≥ 4-5 mm, and 4 = periodontal pockets ≥ 6 mm (Ainamo & Bay 1975). VPI, BOP (Ainamo & Bay, 1975), and periodontal pocket depths (PPD) were measured (Armitage 2004). Caries status was recorded clinically together with bite-wing x-rays.

VPI and root calculus (RC) were recorded from the index teeth (DD 16, 11, 24, 36, 41, 44), and BOP values were recorded for all teeth. PPD was measured for every tooth, but documented only if the values were ≥ 4 mm according to the following scheme: PPD1: one ≥ 4 mm pocket, PPD2: two ≥ 4 mm pockets, and PPD3: three or more ≥ 4 mm pockets. Periodontal measurements were recorded at four sites of teeth.

Based on a questionnaire, details about general health and health habits (e.g. smoking) were registered. Clinical examinations and the aMMP-8 chairside test were performed by the same experienced periodontist.

### 4.1.2 DNA analyses of subclinical periodontitis (Study I) and dental caries (Study II)

Immediately after collection, whole stimulated saliva samples were centrifuged (1,000 × g) for 5 min and saliva supernatants were frozen at –20°C and kept at −70°C until DNA extraction. DNA was extracted from 300 µl of the saliva using a genomic QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotyping was performed using the Agena MassARRAY SNPgenotyping system and iPLEX Gold assays (Agena Biosence, San Diego, Ca, USA) (Heikkinen et al. 2016b). DNA was genotyped for 71 polymorphisms in 29 candidate genes for periodontitis and for 63 polymorphisms in 26 candidate genes for dental caries.

Genetic variants for genotyping were selected from the genes based on previous publications (periodontitis): *S100A8, FCGR2A, PTGS2, IL10, MMP8, MMP3, MMP13, VDR, TLR4, MMP2, MPO, ELANE, IL1A, IL1B, IL1RN, CD28, MMP9, DDX39B, NFKBIL1, LTA, TNF, SOD2, IL6, TLR4, TIMP1*, *SYN1, NCR3, TNFSFI I*, and *Intergenic Chr. 6*.

Genetic variants for genotyping were selected from the genes based on previous publications (dental caries): *S100A8, FCGR2A, FCGR2B, IL10, MMP8, MMP3, MMP13, VDR, TLR4, MMP2, MPO, ELANE, IL1A, IL1B, IL1RN, CD28, MMP9, DDX39B, NFKBIL1, LTA, TNF, SOD2, IL6, TLR4, TIMP1*, and *SYN1*.

### 4.1.3 Subclinical periodontitis and proinflammatory biomarkers (Study III)

Stimulated saliva samples were handled as described in Section 4.1.2.

On the day of analysis, frozen saliva was tested for TREM-1 and PGLYRP1 by commercial enzymelinked immunosorbent assay (ELISA) kits (Nylund et al. 2018). aMMP-8 concentrations were analyzed by a time-resolved immunofluorometric assay (IFMA) (Hanemaaijer et al. 1997). Salivary PMN elastase was measured as an increase in optical density (OD) units due to degradation of synthetic peptide substrate (1 mM SAAVNA), as described earlier (Nieminen et al. 1993).

Adolescents were clustered as periodontally healthy, gingivitis, or subclinical periodontitis. The groups were formed in the following way: healthy group if full-mouth BOP < 20% without deep

periodontal pockets, gingivitis group if BOP ≥ 20% without deep periodontal pockets, and subclinical periodontitis group if subjects had deep periodontal pockets. A periodontal pocket was considered deep if it was ≥ 4 mm.

### 4.2 Study population IV

Study IV was carried out at the Hämeenlinna Health Center in Southern Finland in 2017-2018 among adolescents in eighth grade (14-15 years old). According to a Government Decree (338/2011), in Finland all students in first, fifth and eighth grade are invited to an oral examination. We selected the eighth grade for this study because we considered students of the first and fifth grades to be too young. Altogether 125 participants (n = 65 refused to participate in this study or they did not come to the clinical examination) who gave their consent to the study and the mouth rinse analyses. Adolescents were randomly divided into control group (n = 55; 27 girls and 28 boys) and test group (n=70; 29 girls and 41 boys). The participants were arranged in alphabetical order. Every second participant was selected for the test group, and the remainder comprised the control group. The aMMP-8 test was performed only for the test group. The participants and their parents (one of them) gave written informed consent. Adolescents in orthodontic treatment took part in the study and were included in the study sample. The assumption was that in the control group the prevalence of subclinical periodontitis is 15% and in the test group 32%; p-value (type I error) was set to 5%, and power 80%. Using these assumptions, sample size was n = 94 in both groups. This study was approved by the city of Hämeenlinna and the Ethics Committee of the Helsinki and Uusimaa Hospital District (HUS 1770/2017).

#### 4.2.1 Questionnaire

Participants were asked to fill in a questionnaire comprising questions on oral hygiene habits, snacking, tobacco products, and alcohol, drug and snuff use. Questionnaires were sent together with the suggested appointment time to allow subjects to fill them in at home. Questionnaires were checked before the examination with the oral hygienist. The answers were registered in the researcher's datafile.

#### 4.2.2 Clinical examination

All participants were examined and findings were recorded as in Studies I-III, with a few exceptions.

A radiographic examination was not included. PPD was measured for every tooth and at six sites. Two oral hygienists examined adolescents, and if necessary, forwarded the adolescent for further treatment to a dentist.

### 4.2.3 Mouth rinse collection for the aMMP-8 test

The aMMP-8 chairside test was collected from the test group. If testing positive, the test was taken again after four months. Mouth rinse sampling and scoring of results were performed by an oral hygienist. The oral hygienist coded test positives as slightly positive or positive (+ or ++) or test negatives (-). The results were recorded as 1 (-), 2 (+), and 3 (++). During analyses test positives (+ and ++) were combined. Collected samples were frozen for possible later use.

The aMMP-8 chairside test is based on the immunochromatography principle involving two highly specific monoclonal antibodies (Hanemaaijer et al. 1997).

Briefly: Participants rinse with tap water for 30 seconds and spit it out, then wait for one 1 minute. They rinse with 5 ml of the test solution for 30 seconds. This sample is spat back to the cup and the sampled solution is taken with a syringe. Three drops of the sample solution are dropped into the test draw. The test works on the basis of a lateral flow sandwich immunoassay. The mouth rinse flows towards two antibodies on the test draw. If the mouth rinse contains aMMP-8, it binds to the first antibody and the particles flow along the test draw to the other antibody, which acts as the detecting label (Mäntylä 2006).

The result is provided in 5 minutes and visually graded as positive or negative. The test draw can have up to two lines. One line means (test result identified as 1113 below) that the test is negative, but correctly done, while two lines (test result identified as 1111 below) means that the test is positive (Figure 3). The test is similar to pregnancy test.

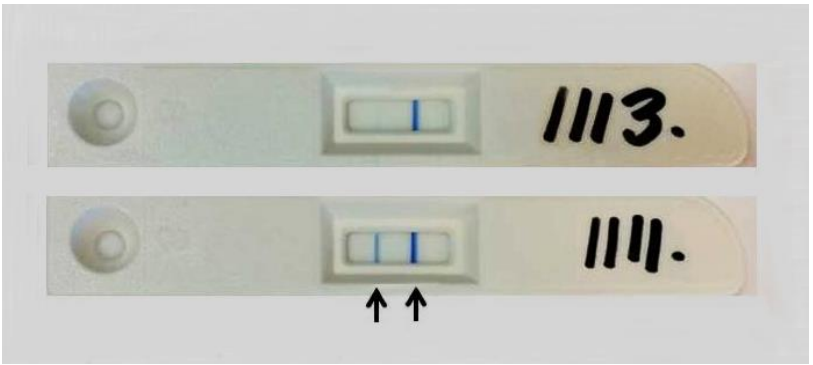


**Figure 3**. Examples of the aMMP-8 mouth rinse test results on the test draws.

#### 4.2.4 Information to the adolescents and the treatment

All participants received oral hygiene instructions. The intervention, which informed adolescents about the effects of tobacco products and alcohol use, teeth cleaning and snacking, was enhanced in both groups. Periodontal treatment involved scaling and professional cleaning. The test group received intensified instructions. The oral hygienist showed the test result for motivation and informed about periodontal diseases. The follow-up time for test-positive participants was four months. If testing positive after the first follow-up session, the test was taken again after four months.

Detailed materials and methods are described in the original publications I-IV.

### 4.3 Statistical analyses

Significance was set at $p < 0.05$ values. P-values were corrected for multiple testing using false discovery rate (FDR) in Study I and II (Benjamini & Hochberg 1995, Glickman et al. 2014).

In **Study I**, associations between aMMP-8 chairside test results and PPD3-positive (three or more ≥ 4 mm deepened periodontal pockets) participants, and between aMMP-8 chairside test results and those participants with restorative treatment need ($D > 0$) and the SNPs were analysed by means of a logistic regression model using R language (R Core Team 2017).

In **Study II**, logistic regression model was used to investigate the association between decayed and initial caries teeth and SNPs. An unadjusted model (SNP as the only explanatory variable) and an adjusted model (VPI, weekly smoking, and study periods) were calculated. All data analyses were

carried out using R software version 3.2.2. (R Core Team 2017) and SNPassoc package (González et al. 2007).

In **Study III**, descriptive statistics (mean, median, min, max, quartiles, and standard deviations) and correlation analyses between clinical parameters or between clinical parameters and salivary analytes (Wilcoxon rank-sum test) were calculated using R software version 3.4.1. GraphPad Prism 7 statistical software (GraphPad Software, Inc., La Jolla, CA, USA) was used to determine differences in analyte levels between different groups (healthy, gingivitis, subclinical periodontitis) by applying the Kruskal-Wallis test (non-parametric), and the Spearman rank correlation test (group independent) was applied to evaluate correlations between the four different analytes (TREM-1, PGLYRP1, aMMP-8, and PMN elastase). Receiver operating characteristic curves (ROC) were constructed to assess the ability of PGLYRP1 as a diagnostic aid.

In **Study IV**, oral health habits and usage of tobacco products, alcohol, and drugs were compared between the test and control groups, and between boys and girls by Fisher's Exact Test. Differences in periodontal parameters between the groups and between the aMMP-8 mouth rinse test positives and negatives were tested using the Mann-Whitney U-test. The Friedman test was used for comparing the treatment effect at three time points (baseline, four months, eight months). Pairwise post hoc testing was calculated by the Dunn-Bonferroni test. The treatment effect among the adolescents with two visits (baseline, four months) was tested with the Wilcoxon rank-sum test. Data analysis was performed using IBM SPSS Statistics, version 25.

## 5. RESULTS

### 5.1 Genetic background of subclinical periodontitis (Study I)

Of the 47 participants, 13 (27.7%) were aMMP-8 chairside test positive. Only five were regular smokers. Two of the smokers were test positive and had three or more ≥ 4 mm pockets (PPD3). In DNA analyses, one person was excluded because of a discrepant sex check, and thus, the final number of participants in DNA analysis was 46. Participants who were aMMP-8 chairside test and PPD3 positive showed an association with SNPs in *VDR* (rs2228570, p = 0.002, q = 0.04) and *MMP3* (rs520540, rs639752, rs679620, p = 0.0009, 0.003, 0.003, q = 0.04, respectively). None of the other SNPs showed a significant association with aMMP-8 chairside test positivity and at least one caries lesion restorative treatment need (D > 0).

### 5.2 Genetic background of dental caries (Study II)

Of the 94 participants, 30 (31.9%) had at least one caries lesion and 57 (60.6%) had none. Data were missing from seven participants. Sixty participants (63.8%) had at least one initial caries lesion, 28 participants did not have any, and data were missing for six participants. Of all participants, 16 (17.0%) were regular smokers. In DNA analyses, one person was excluded because of a discrepant sex check, and thus, the final number of participants in DNA analysis was 93. SNPs in *DDX39B* (rs7766569, p = 0.03, q = 0.688885) and *MPO* (rs2243828, p = 0.04, q = 0.688885) showed association tendencies with caries lesions. However, the outcome did not remain significant when corrected for multiple testing by false discovery rate (FDR). Other studied SNPs did not show any associations. SNPs in *VDR* (rs2228570, p = 0.01, q = 0.50), *LTA* (rs2009658, p = 0.02, q = 0.50), and *MMP3* (rs650108, p = 0.03, q = 0.61) showed no significant associations after FDR with initial caries lesions.

### 5.3 Presence of salivary proinflammatory biomarkers, aMMP-8, TREM1, PGLYRP-1, and PMN elastase, in association with subclinical periodontitis (Study III)

Of the participants, 536 (one was excluded because of unavailable measurements) were divided into three groups according to their periodontal health status: healthy group (H), gingivitis group (G), and subclinical periodontitis group (sP) (Table 2). In the healthy group, mean age was 15.46 ± 0.81 years, in the gingivitis group 15.07 ± 0.25 years, and in the subclinical periodontitis group 15.15 ± 0.42 years. Adolescents with gingivitis or subclinical periodontitis were younger (4 months, 3 months, respectively) than those with a healthy periodontium (p < 0.0001, p = 0.0002, respectively). There were no significant differences between the groups (p > 0.05) when assessed by sex. VPI and BOP

were significantly higher in adolescents with subclinical periodontitis or gingivitis than individuals with a healthy periodontium ($p < 0.0001$, $p < 0.0001$, respectively).

**Table 2**. Distribution of participants.

| Groups | Girls | Boys | Total |
|---|---|---|---|
| Healthy group | 16 | 10 | **26 (4.8%)** |
| Gingivitis group | 96 | 70 | **166 (31.0%)** |
| Subclinical group | 143 | 201 | **344 (64.2%)** |
| **Total** | **255 (47.6%)** | **281 (52.4%)** | **536 (100.0%)** |

TREM-1, PGLYRP1, aMMP-8, and PMN elastase were detectable in more than 90% of all saliva samples. The level of salivary PGLYRP1 was significantly higher in adolescents with subclinical periodontitis and gingivitis than in individuals with a healthy periodontitis. The level of aMMP-8 was significantly higher in subclinical periodontitis group than in gingivitis or subclinical periodontitis groups. (Figure 4, Table 3). TREM-1 levels were higher, but not significantly, in adolescents with subclinical periodontitis than in individuals with gingivitis or a healthy periodontium (Figure 4, Table 3). The median concentrations of PMN elastase were also higher, but not significantly, in adolescents with subclinical periodontitis than in individuals with gingivitis or a healthy periodontium (Figure 4, Table 3).

**Table 3.** Salivary analytes in healthy (H), gingivitis (G), and subclinical periodontitis (sP) groups (n=536 [a]).

| | | Mean | Median | sd | Min | Max | n | N/A | p-value |
|---|---|---|---|---|---|---|---|---|---|
| PGLYRP1 (pg/ml) | H | 9956.1 | 6089.5 | 10038.3 | 659.5 | 39412.9 | 26 | 0 | reference |
| | G | 15517.9 | 12015.8 | 11079.3 | 1344.8 | 55922.4 | 166 | 0 | **0.0374** |
| | sP | 17397.9 | 12948.0 | 13479.8 | 29.2 | 63724.5 | 344 | 0 | **0.0039** |
| TREM-1 (pg/ml) | H | 352.5 | 184.3 | 337.5 | 22.0 | 1194.0 | 26 | 0 | reference |
| | G | 406.0 | 343.9 | 318.2 | 15.4 | 1908.0 | 166 | 0 | 0.4760 |
| | sP | 440.3 | 333.1 | 373.6 | 1.9 | 2411.0 | 344 | 0 | 0.2250 |
| aMMP-8 (ng/ml) | H | 139.9 | 123.8 | 100.1 | 14.7 | 406.2 | 26 | 0 | reference |
| | G | 205.2 | 138.2 | 214.9 | 5.3 | 1217.5 | 166 | 0 | 0.1704 |
| | sP | 243.9 | 187.1 | 236.8 | 2.4 | 1485.0 | 343 | 1 | **0.0237** |
| PMNelastase | H | 51.7 | 7.0 | 86.7 | 1.9 | 281.1 | 11 | 15 | reference |
| (ΔOD405/h) | G | 22.4 | 9.9 | 40.8 | 0.71 | 375.6 | 166 | 0 | 0.1809 |
| | sP | 37.9 | 10.2 | 81.0 | 0.25 | 808.5 | 316 | 28 | 0.5213 |

H = healthy, G = gingivitis, sP = subclinical periodontitis, N/A = not applicable, PGLYRP1 = peptidoglycan recognition protein 1, TREM-1 = triggering receptor expressed on myeloid cells 1, aMMP-8 = active matrix metalloproteinase 8, PMN elastase = polymorphonuclear elastase

[a] one value missing for healthy/gingivitis/subclinical periodontitis groups

p-values were obtained using general linear model (significant values are depicted in bold)

p-values less than 0.05 were considered significant

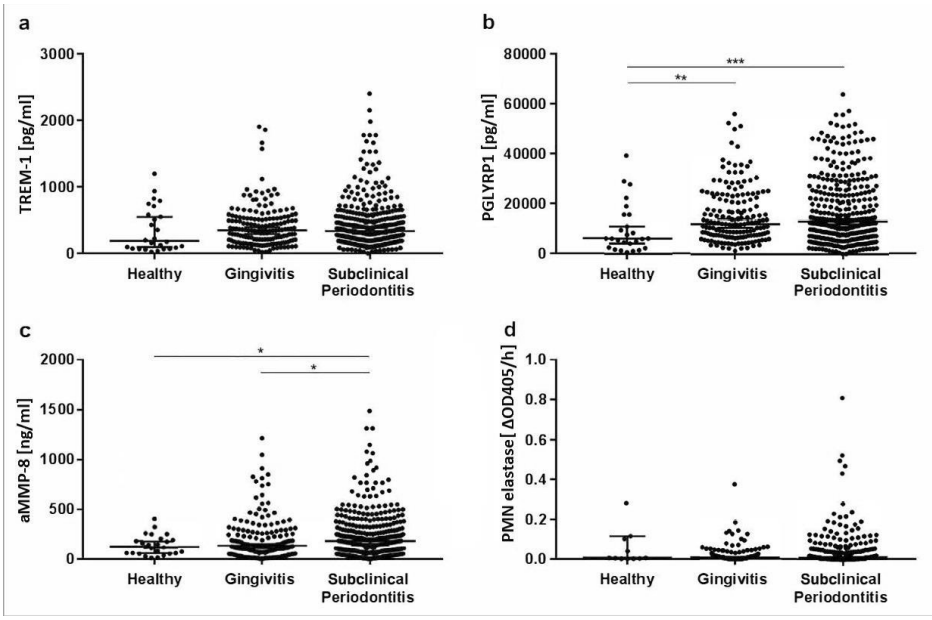


**Figure 4.** Salivary levels of TREM-1, PGLYRP1, aMMP-8, and PMN elastase.
PGLYRP1 = peptidoglycan recognition protein 1, TREM-1 = triggering receptor expressed on myeloid cells 1, aMMP-8 = active matrix metalloproteinase 8, PMN elastase = polymorphonuclear neutrophil elastase
Concentrations of TREM-1 (a, pg/ml), PGLYRP1 (b, pg/ml), aMMP-8 (c, ng/ml), and PMN elastase (d, ΔOD405/h) in saliva are indicated by single values grouped into healthy, gingivitis, and subclinical periodontitis
Horizontal lines indicate median values (± 95% CI)
*Reprinted with permission from Sage journals. Raivisto et al. Regulation of Salivary Peptidoglycan Recognition Protein 1 in Adolescents. JDR Clin Trans Res 2019:2380084419894287.*

A positive significant correlation ($p < 0.0001$) between all tested analyte combinations – TREM-1, PGLYRP1, aMMP-8, and PMN elastase – was demonstrated. The strongest correlation was found for TREM-1/PMN elastase ($r = 0.7024$, $p < 0.0001$), followed by PGLYRP1/aMMP-8 ($r = 0.6322$, $p < 0.0001$), TREM-1/PGLYRP1 ($r = 0.6238$, $p < 0.0001$), TREM-1/aMMP-8 ($r = 0.5879$, $p < 0.0001$), aMMP-8/PMN elastase ($r = 0.5871$, $p < 0.0001$), and PGLYRP1/PMN elastase ($r = 0.5758$, $p < 0.0001$). Receiver operating characteristic analysis showed an area under the curve (AUC) of 0.55 (95% CI 0.50-0.60) for diagnosis of subclinical periodontitis.

Levels of aMMP-8 > median 161.9 ng/ml and > mean 150.0 ng/ml and PMN elastase > median 0.01 ΔOD405/h correlated positively with TREM-1 and PGLYRP1 levels in saliva (both $p < 0.0001$). Adolescents with VPI > median 45.8% showed an association with PGLYRP1 ($p = 0.0182$) and TREM-1 ($p = 0.0246$) levels. Both PGLYRP1 and TREM-1 levels were elevated among BOP scores

≥ 20% ($p < 0.0001$, $p = 0.0803$, respectively) (Figure 5). The level of PGLYRP1 was significantly higher in the PPD2 group ($p = 0.0109$). Neither TREM-1 nor PGLYRP1 significantly correlated with smoking. Girls had higher levels of PGLYRP1 ($p = 0.0005$) and TREM-1 ($p = 0.1630$) than boys, but the latter did not reach significance.

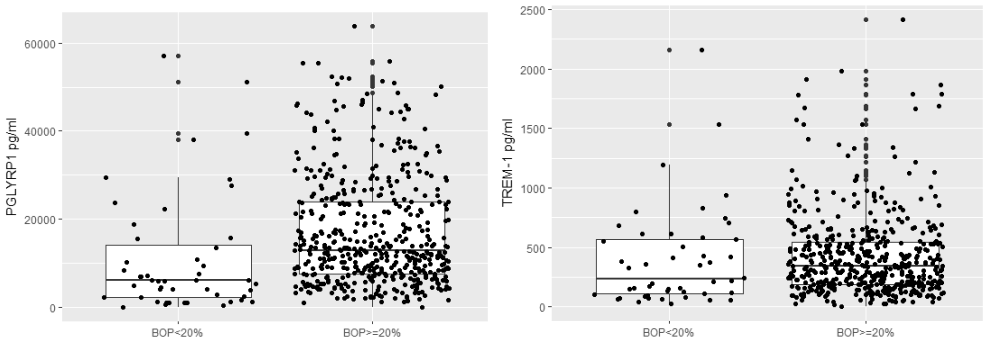


**Figure 5**. BOP scores ≥20 % associated with PGLYRP1 and TREM-1.

PGLYRP1 = peptidoglycan recognition protein 1 (pg/ml), TREM-1 = triggering receptor expressed on myeloid cells 1 (pg/ml), BOP = bleeding on probing

### 5.4 aMMP-8 test in association with periodontal parameters, dental caries, oral health behaviours. and effects of treatment and preventive intervention (Study IV)

#### 5.4.1 Periodontal parameters

Of the 70 adolescents in the test group, 24 tested positive (34.3%). Associations between the test and periodontal parameters are shown in Table 4. VPI% was significantly higher in test positives ($p = 0.005$) than test negatives.

**Table 4**. Association between aMMP-8 mouth rinse test and periodontal parameters.

| | aMMP-8 mouth rinse test | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Test positives n=24 | | | | Test negatives n=46 | | | | |
| | Min | Max | Median (IQR) | Missing (n) | Min | Max | Median (IQR) | Missing (n) | p-value |
| BOP% | 0 | 16.00 | 6.70 (2.01-10.71) | 0 | 0 | 21.00 | 3.57 (0.45-7.59) | 1 | 0.052 |
| VPI% | 0 | 37.50 | 6.25 (1.04-12.50) | 0 | 0 | 41.67 | 0.00 (0.00-4.17) | 1 | **0.005** |
| RC% | 0 | 37.50 | 4.17 (0-15.63) | 0 | 0 | 37.50 | 12.50 (4.17-12.50) | 1 | 0.131 |
| PPD | 0 | 3 | 0 (0) | 0 | 0 | 3 | 0 (0) | 1 | 0.895 |
| CPITN | 1 | 3 | 2 (1-2) | 2 | 0 | 3 | 2 (1-2) | 0 | 0.693 |

aMMP-8 = active matrix metalloproteinase-8
IQR = interquartile range
BOP% = bleeding on probing percentage, VPI% = visible plaque percentage, RC% = root calculus percentage
PPD = number of at least 4 mm deepened periodontal pockets
CPITN max = highest CPITN score in six sextants
p-value = Mann Whitney U-test (2-sided)

Five adolescents in the test group and two in the control group had deepened periodontal pockets (Table 5). Of those in the test group, two had a positive and three a negative test result.

**Table 5**. Deepened periodontal pocket depths (PPD ≥ 4 mm).

| Group | PPD1 | PPD2 | PPD3 | Total |
|---|---|---|---|---|
| **Test group** | 2 | 1 | 2 | 5 |
| **Control group** | | | 2 | 2 |

PPD1 = one ≥ 4 mm deepened periodontal pocket, PPD2 = two ≥ 4 mm deepened periodontal pockets, PPD3 = three or more ≥ 4 mm deepened periodontal pockets

The highest CPITN score was mostly 1 to 2 (88.8%). Of the participants, 29.6% did not have any healthy sextants. Girls had significantly more healthy sextants than boys (p = 0.001); however, there was no significant difference between the test and control groups or between test positives and test negatives. Highest CPITN score did not differ between test positives and test negatives (p = 0.278).

### 5.4.2 Dental caries

Of the participants 23 (18.4%) had one to two caries lesions and 92 (73.6%) did not have any. Ten adolescents (8.0%) had three or more caries lesions. DMF score was 0 in 48.0% of adolescents. Forty-two (33.6%) had DMF score 1 to 2 and 23 (18.4%) 3 or more. Thirty-eight (30.4%) did not have any initial caries lesions, 36 (28.8%) had one to two and 50 (40.0%) three or more initial caries lesions. Data were missing for one participant (0.8%). DMF, D or i values did not differ between girls and boys or between aMMP-8 test-positive and test-negative participants.

### 5.4.3 Oral health behaviours

Of the participants, most brushed their teeth at least twice a day, with girls brushing their teeth significantly more often than boys. Interdental spaces were cleaned quite randomly. Adolescents ate candies mostly 1-2 times a week, and boys drank more often soft drinks than girls. These results are summarized in Table 6 (Raivisto et al. unpublished results).

Of all participants, 111 (88.8%) had never smoked. Only four (3.2%) were current smokers. Seven had experimented with electro tobacco, but no one smoked it regularly. Most of the participants had never used snuff (76.8%), but eight had experimented with it. Only one girl (in the test group) was a snuff-user. Of the boys, 62 (49.6%) and of the girls, 48 (38.4%) had never consumed alcohol, 11 (8.8%) adolescents had experimented with alcohol. No one used or had experimented with illegal drugs.

**Table 6**. Oral health behaviours in the test and control groups, and between boys and girls, and according to the aMMP-8 test results.

| | **Test Group** (n=70) n (%) | | | | **Control Group** (n=55) n (%) | | p-value | p-value |
|---|---|---|---|---|---|---|---|---|
| | Boys | Girls | aMMP-8 +[a] | | Boys | Girls | Test vs. control group | Boys vs. girls |
| **Toothbrushing** | | | Boys | Girls | | | | |
| **Less than once a day** | 2 (4.9) | 0 | 1 (5.3) | 0 | 4 (14.3) | 0 | 0.396 | **0.002** |
| **Once a day** | 10 (24.4) | 2 (6.9) | 5 (26.3) | 0 | 9 (32.1) | 5 (18.5) | | |
| **Twice a day** | 23 (56.1) | 24 (82.8) | 11 (57.9) | 5 (100.0) | 11 (39.3) | 20 (74.1) | | |
| **Once or twice a day** | 6 (14.6) | 3 (10.3) | 2 (10.5) | 0 | 4 (14.3) | 2 (7.4) | | |
| **Missing** | 0 | 0 | 0 | 0 | 0 | 0 | | |
| **Dental space cleaning** | | | | | | | | |
| **Not at all** | 6 (14.7) | 3 (10.3) | 5(26.3) | 2 (40.0) | 2 (7.2) | 0 | 0.342 | 0.354 |
| **Less than once a day** | 31 (75.6) | 20 (69.0) | 12 (63.2) | 2 (40.0) | 23 (82.1) | 23 (85.2) | | |
| **Once a day** | 2 (4.9) | 5 (17.2) | 2 (10.5) | 1 (20.0) | 3 (10.7) | 3 (11.1) | | |
| **Twice a day** | 1 (2.4) | 1 (3.5) | 0 | 0 | 0 | 1 (3.7) | | |
| **Missing** | 1(2.4) | 0 | 0 | 0 | 0 | 0 | | |
| **Candies** | | | | | | | | |
| **Not often** | 6 (14.6) | 2 (6.9) | 1 (5.3) | 0 | 4 (14.3) | 2 (7.4) | 1.000 | 0.572 |
| **1-2 times a week** | 30 (73.2) | 26 (89.7) | 18 (94.7) | 5 (100.0) | 22 (78.5) | 22 (81.5) | | |
| **Many times a week** | 3 (7.4) | 1 (3.4) | 0 | 0 | 1 (3.6) | 3 (11.1) | | |
| **Every day** | 1 (2.4) | 0 | 0 | 0 | 1 (3.6) | 0 | | |
| **Missing** | 1 (2.4) | 0 | 0 | 0 | 0 | 0 | | |
| **Soft drinks** | | | | | | | | |
| **Not often** | 10 (24.4) | 15 (51.8) | 2 (10.5) | 1 (20.0) | 6 (21.4) | 15 (55.6) | 0.915 | **0.0003** |
| **1-2 times a week** | 22 (53.7) | 12 (41.4) | 13 (68.4) | 4 (80.0) | 15 (53.6) | 10 (37.0) | | |
| **Many times a week** | 5 (12.2) | 1 (3.4) | 3 (15.8) | 0 | 3 (10.7) | 1 (3.7) | | |
| **Every day** | 3 (7.3) | 0 | 1 (5.3) | 0 | 4 (14.3) | 0 | | |
| **Missing** | 1 (2.4) | 1 (3.4) | 0 | 0 | 0 | 1 (3.7) | | |
| **Snacks** | | | | | | | | |
| **Not often** | 13 (31.7) | 10 (34.5) | 9 (47.4) | 2 (40.0) | 4 (14.3) | 2 (7.4) | **0.002** | 0.788 |
| **1-2 times a week** | 8 (19.5) | 8 (27.6) | 3 (15.8) | 1 (20.0) | 4 (14.3) | 3 (11.1) | | |
| **Many times a week** | 6 (14.6) | 6 (20.7) | 2 (10.5) | 1 (20.0) | 6 (21.4) | 7 (25.9) | | |
| **Every day** | 12 (29.3) | 4 (13.8) | 5 (26.3) | 1 (20.0) | 13 (46.4) | 13 (48.2) | | |
| **Missing** | 2 (4.9) | 1 (3.4) | 0 | 0 | 1 (3.6) | 2 (7.4) | | |

[a] aMMP-8 + = a positive aMMP-8 test result
p-values = Fisher's exact test (2-sided)

### 5.4.4 Effects of treatment and preventive intervention

The aMMP-8 levels decreased after periodontal treatment intervention by a change in test stick result from positive (+) to negative (-) during follow-up. Of the 24 participants testing positive, 11 (46%) had one treatment period and 9 (38%) had two treatment periods before their test result turned negative. Three adolescents did not visit the oral hygienist again after the examination and aMMP-8 mouth rinse test. Only one adolescent was test positive after two treatment periods. The effects of treatment on BOP%, VPI% and RC% are shown in Figure 6. Adolescents with one treatment period had significantly lower levels of VPI and RC after the treatment period (Table 7). VPI and RC levels were lower also among adolescents with two treatment periods, although not significantly. BOP levels were also reduced, but not significantly, in both groups with one or two treatment periods. Of participants, 25 (15 in test group and 10 in control group) were in active orthodontic treatment (fixed or removable appliances) during this study. Five of them in the test group were test positives, but changed to negative during periodontal treatment.

**Table 7**. Adolescents with one treatment period before the aMMP-8 mouth rinse test result turned from positive to negative. Follow-up time was four months.

| | | **Before treatment** | | **After treatment** | | |
|---|---|---|---|---|---|---|
| | N | Mean | Median (IQR) | Mean | Median (IQR) | p-value |
| BOP% | 11 | 6.65 | 6.25 (2.68-10.27) | 4.38 | 4.46 (2.23-5.80) | 0.229 |
| VPI% | 11 | 13.63 | 8.33 (6.25-18.75) | 2.29 | 0.00 (0.00-4.17) | **0.011** |
| RC% | 11 | 8.33 | 4.17 (0.00-10.42) | 3.42 | 0.00 (0.00-2.08) | **0.031** |

IQR = interquartile range
BOP% = bleeding on probing percentage, VPI% = visible plaque percentage, RC% = root calculus percentage
p-value = Wilcoxon-test, Exact Sig. (2-tailed)

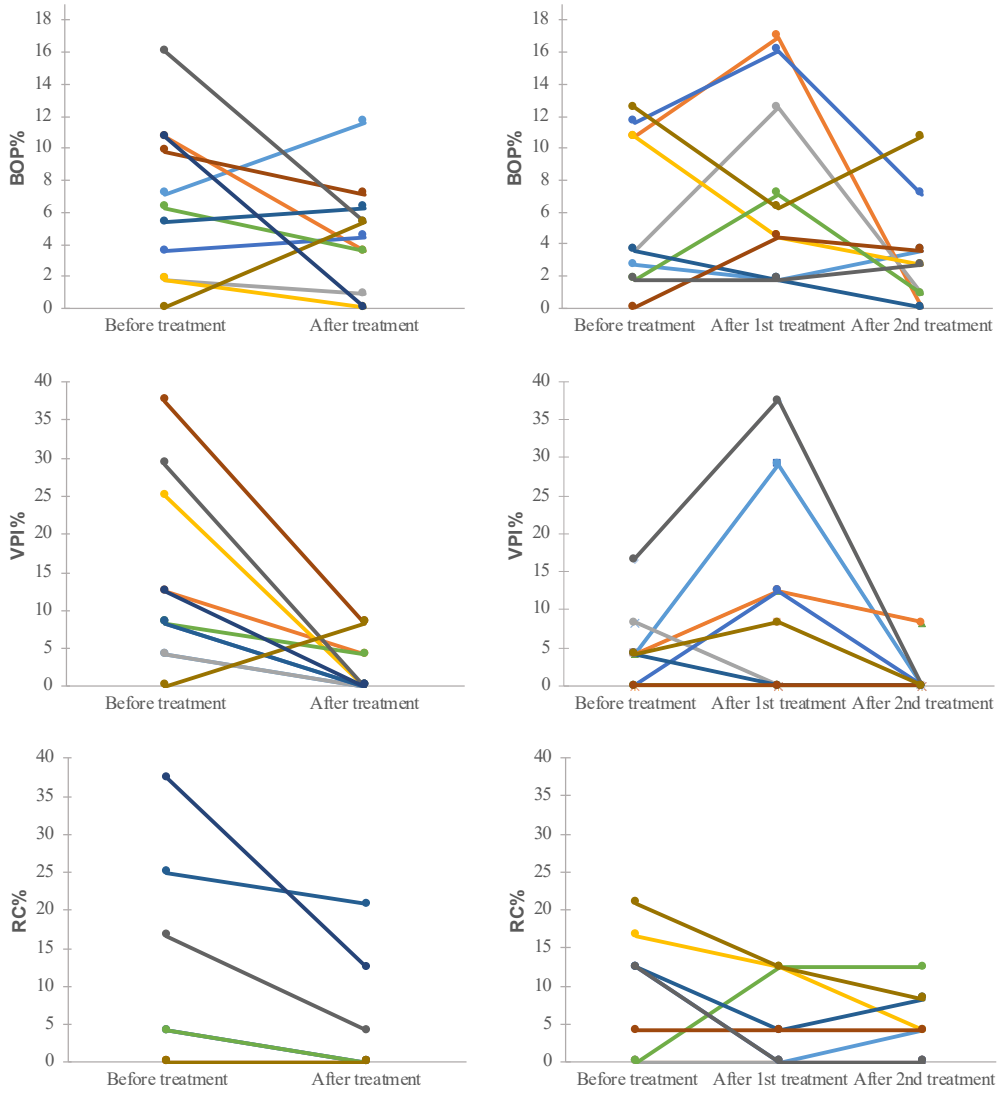


**Figure 6**. Effect of treatment on BOP%, VPI% and RC%.
BOP% = bleeding on probing percentage, VPI% = visible plaque percentage, RC% = root calculus percentage Percentages related to the aMMP-8 chairside mouth rinse test outcomes during the intervention treatment period. *Reprinted with permission from JCDR. Raivisto et al. Active Matrix Metalloproteinase-8 Chair Side Mouth Rinse Test, Health Behaviour and Oral Health in Finnish Adolescent Cohort. JCDR 2020; 14:ZC35-ZC39*

## 6. DISCUSSION

The main finding in our study was that the aMMP-8 mouth rinse test could identify adolescents with poor oral hygiene at risk for subclinical periodontitis. An oral health preventive intervention, such as oral health behaviour instructions combined with periodontal treatment, decreased aMMP-8 levels. We also found that aMMP-8 chairside test positivity with deep pockets was associated to genetic polymorphisms of *MMP3* and *VDR* genes.

### 6.1 Subclinical periodontitis and SNPs (Study I)

We studied the genetic background of subclinical periodontitis in adolescents. Genetics could explain about 50% of the susceptibility to periodontitis (Laine et al. 2012). We found that aMMP-8 chairside test positivity with deep pockets was linked to *MMP3* and *VDR* genes. Our results confirm previous findings of an association with genetic variations in *MMP3*. *MMP3* showed an association with chronic periodontitis in the US population (rs679620 and rs 650108) and in the Brazilian population (rs 639752) (Letra et al. 2012). The *MMP3* gene showed that the genotype 5A/5A was significantly more frequent in Brazilian adults who suffered from periodontitis, whereas *MMP1* polymorphism had no significant association with periodontitis (Astolfi et al. 2006).

### 6.2 Dental caries and SNPs (Study II)

In our study, association tendencies between the studied SNPs in *DDX39B* and *MPO* and dental caries as well as between SNPs in *VDR*, *LTA*, and *MMP3* and initial caries lesions were found. However, none of these associations remained significant after FDR correction.

In the literature, the heritability of dental caries varies between 40% and 60% (Wang et al. 2010; Wendell et al. 2010; Shungin et al. 2019). Several genes are involved in enamel formation (*AMEX*, *AMBN*, *ENAM*, *TUFT*, *MMP20*, and *KLK4*) (Lewis et al. 2017). Adolescents at risk for dental caries could be identified by genes, and implementation of preventive strategies could be allocated before the development of caries (Wendell et al. 2010).

An interesting observation is that genes affecting susceptibility to caries in the primary dentition may differ from those in permanent teeth (Wang et al. 2010). *VDR* gene polymorphisms have been shown to have potential as a marker for the identification of children with high caries risk. However, in our study SNPs in the *VDR* gene showed no significant associations. Cogulu et al. (2016) found a statistically significant difference in the frequency of *VDR TaqI* genotypes (tt) between caries-active and caries-free children, but not between *VDR ApaI*, *FokI* and *Cdx2* genotypes. The risk of dental

caries was higher with the heterozygous *VDR TaqI* genotype (tt) also according to Hu et al. (2015). The *VDR-FokI* gene polymorphism has been associated with dental caries in children in China. However, *VDR* gene polymorphisms (*BsmI*, *TaqI*, and *ApaI*) showed no significant differences in the caries group compared with the controls (Yu et al. 2017). Also in a Czech study, the *VDR TaqI* gene variant could not be used as a marker for identifying children with increased dental caries risk (Izakovicova et al. 2017).

We did not find an association between the studied *MMP2*, *MMP3*, *MMP8*, *MMP9*, and *MMP13* SNPs and caries. Regardless, the importance of *MMP*s in the development and progression of dentin caries has been demonstrated by numerous other studies. *MMP9* and *MMP20* were involved in white spot lesions and early childhood caries development (Antunes et al. 2016). Associations with dental caries have been found in *MMP10*, *MMP14*, *MMP16* (Lewis et al. 2017), and *MMP13* (Tannure et al. 2012). *MMP2* and *MMP3* genes are likely to be involved in caries (Menezes-Silva et al. 2012; Karayasheva et al. 2016).

**6.3 Subclinical periodontitis and proinflammatory biomarkers (Study III)**

We found a strong correlation between salivary PGLYRP1 levels, VPI%, and BOP%, indicating that adolescents with clinical signs of gingival inflammation (BOP ≥ 20%) and risk for periodontitis were showing higher levels of PGLYRP1 in saliva. PGLYRP1 is implicated in the pro-inflammatory response towards bacterial infections (Bleharski et al. 2003) and has been identified as a functional ligand for TREM-1 (Read et al. 2015). Bacteria bind to TREM-1 through PGLYRP1, unlike other TREMs, which bind directly to bacterially derived components (Read et al. 2015). Thus, it is not surprising that PGLYRP1 correlated positively with TREM-1. Several studies have reported a TREM-1 association with periodontal inflammation (Bostanci et al. 2013b; Belibasakis et al. 2014; Willi et al. 2014) Periodontal pathogens modulate the TREM-1 signaling pathway (Bostanci et al. 2011; Bostanci & Belibasakis 2012). Our findings with adolescents are in line with the above studies.

In our study, PGLYRP1 also correlated strongly with aMMP-8 and PMN elastase. This might be explained by all molecules deriving from the same type of immune cells and being released upon neutrophil degranulation (Dziarski & Gupta 2006). PMN elastases are components of PMN granules involved in bacterial defence mechanisms (Alfakry et al. 2016). PMN elastase also activates MMPs. A positive association between PGLYRP1, TREM-1, aMMP-8, and PMN elastase has also been found by Nylund et al. (2018). Especially PMN-released aMMP-8 has been associated with

periodontitis in adults (Rathnayake et al. 2013) and with subclinical periodontitis in adolescents (Heikkinen et al. 2008). Our findings strengthen these results.

In our study, female adolescents had significantly higher levels of PGLYRP1 than males, but levels of TREM-1 showed no such association. Shiau & Reynolds (2010) published in their study that sex steroids have an essential role in periodontitis, and it is well known that menstruation has an influence on gingival inflammation and bleeding (Khosravisamani et al. 2014). In addition, oral contraceptive users have been shown to have a significant association with severe periodontitis (Domingues et al. 2012). PGLYRP1 and TREM-1 are mainly derived from PMNs, with the difference that PGLYRP1 is a soluble protein in PMN granules, while TREM-1 is expressed as a membrane receptor on the PMN surface. Alteration of the chemotactic and phagocytic functions of PMNs by female sex hormones (Miyagi et al. 1992) may explain our results. It might be that levels of PGLYRP1 and TREM-1 are affected differently by these alterations. In our study, neither PGLYRP1 nor TREM-1 was significantly associated with smoking, although smoking is a well-known behavioural risk factor for periodontitis (Leite et al. 2018). Similar results were presented by Bostanci et al. (2013a).

**6.4 aMMP-8 test in adolescents and preventive intervention (Study IV)**

The aMMP-8 mouth rinse test could identify adolescents with poor oral hygiene at risk for subclinical periodontitis mainly without visible manifestations of illness. In this regard, our results are in line with earlier studies among adolescents (Heikkinen et al. 2016a, 2017, 2019). A very strong correlation between aMMP-8 test and oral hygiene index was found among Nigerian adult males (Nwhator et al. 2014). Furthermore, according to Räisänen et al. (2019), the test seemed to be more effective than BOP in detecting subclinical periodontitis in adolescents and more robust to the confounding effects of oral hygiene than BOP (Sorsa et al. 2020). An interesting observation is that caries also correlates strongly with salivary levels of MMP-8 (Hedenbjörk-Lager et al. 2015). However, contrary results have been reported by Schmidt et al. (2018), who failed to find a significant relationship between carious lesions and aMMP-8 ($p > 0.05$). In our study, dental caries, initial caries, and even DMF did not differ between aMMP-8 test positives and negatives.

A positive aMMP-8 test result only occurred for those adolescents who were in need of periodontal treatment (CPITN = 1, 2 or 3). So the test had no false positives when related to CPITN. We wished to be pedantic because we were searching for an early stage of periodontitis, and thus, we also took into account gingivitis. Periodontal diseases refer to both gingivitis and periodontitis (Kinane 2001).

Only seven participants had deepened periodontal pockets (≥ 4 mm). Therefore, only a few adolescents had visible or detectable signs of periodontitis.

Of the 70 adolescents in the test group, 24 tested positive (aMMP-8 > 20 ng/ml). This large proportion is alarming and is consistent with the study by Heikkinen et al. (2016a) among adolescents. In our study, an oral health preventive intervention, such as oral health behaviour instructions combined with periodontal treatment, decreased aMMP-8 levels by changes in test stick results from positive to negative. The reduction has also been demonstrated in other studies (Mäntylä et al. 2003; Heikkinen et al. 2010; Sorsa et al. 2016; Alassiri et al. 2018). Simultaneously, reductions were seen in plaque accumulation and bleeding.

### 6.5 Oral health behaviours and aMMP-8 chairside test (Study IV)

Oral health professionals are in a key position to share knowledge of oral health promotion. Sustainable behavioural change approaches are the aim in preventive efforts. Compared with professional-centred education, the motivational interview of the patient seems to enhance self-care of patients with periodontitis, improving oral health (Järvinen 2020). Changing behaviour is a complex process that takes place over time and often include temporary improvements and lapses (Prochaska 2005). Tolvanen et al. (2012) evaluated the oral health-related knowledge-attitudes-behaviour axis. Tooth brushing seemed to be the most important attitude in influencing oral hygiene behaviour.

In our study, most adolescents (62.4%) brushed their teeth at least twice a day. The same outcome was found with Spanish adolescents. Nearly two-thirds of them (mean age 15.26 years) brushed their teeth more than once a day (Costa-Tutusaus & Guerra-Balic 2016). Girls brushed their teeth more often than boys according to previous studies by Maes et al. (2006), Costa-Tutusaus & Guerra-Balic (2016), and Heikkinen et al. (2008, 2016a). Our findings support this. Bacterial dental plaque does not lead only to dental caries but also to periodontal disease. It is considered to be the major risk factor for the development of periodontal disease (Uitto et al. 2003; Sorsa et al. 2004, 2006). Using tobacco products and alcohol consumption were of minor importance in our study due to the small numbers of regular smokers and alcohol consumers. Compared with the present study population, alcohol consumption (Samuelson 2000) and smoking (Banzer et al. 2017) appear to be common in European adolescents.

In our study, adolescents ate candies and drank soft drinks mostly one to two times a week. Girls have healthier food choices than boys according to Diethelm et al. (2012) and Costa-Tutusaus & Guerra-Balic (2016). Girls may also more often under-report their dietary intake than boys (Diethelm et al. 2012). Boys reported more frequent consumption of soft drinks in our study as well as in another study in Finland among adolescents of the same age (Hoppu et al. 2008). According to the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) cross-sectional survey, girls enjoyed more water, fruit juice, and sweets (Rey-Lopez et al. 2011). Hoppu et al. (2008) also concluded that girls consume more sweets. Improvements are needed with respect to eating habits.

### 6.6 Limitations and strengths of the study

A limitation of the present series of genetic studies is the relatively small sample sizes in Study I, n = 47 and Study II, n = 94. Furthermore, consent is needed from adolescents and their parents if the participants are aged under 15 years. This is challenging considering teenagers' disinterest and the general suspicion towards genetic studies. However, all participants who gave consent were included.

A strength of our study was the large sample sizes in Studies III, n = 537 and IV, n = 125. Moreover, the participants were examined by the same experienced periodontist in the study I-III, and mouth rinses were analysed by the same oral hygienist in the Study IV. Another strength was also a comprehensive oral health examination and adolescents as the target population. Most studies are conducted among adults.

### 6.7 Practical relevance

As mentioned earlier, periodontal diseases and dental caries are the most common inflammatory infection diseases in the world, and according to our studies even adolescents suffer from subclinical periodontitis. Childhood oral infections have recently been shown to be associated with and possibly predict subclinical carotid atherosclerosis in adulthood (Pussinen et al. 2019). Periodontitis has relevance for a general health. Its detection would be cost-effective for society. The aMMP-8 chairside mouth rinse test could be part of clinical examinations to identify and alert adolescents with poor oral hygiene at risk for subclinical periodontitis.

### 6.8 Further research

The aMMP-8 chairside test could detect subclinical periodontitis in adolescents with a predisposing genetic background, however, more studies are needed in this regard. A broader genome-wide scanning periodontal disease SNP analysis with the same study material would be interesting. More

studies with larger sample size are needed before final conclusions can be drawn about genetic factors linked to dental caries. Further studies are also required to evaluate whether salivary PGLYRP1 is affected by the presence of other chronic diseases to judge its ability as a potential periodontal disease marker in the future. The aMMP-8 chairside mouth rinse test with a longer follow-up time and a larger sample of adolescents warrants additional research.

## 7. CONCLUSIONS

Based on our studies the aMMP-8 chairside test could detect subclinical periodontitis in adolescents with a genetic predisposition background, whereas only tentative genetic aetiology for dental caries was observed. It seems that genetic polymorphisms of *MMP3* and *VDR* genes are linked to subclinical periodontitis in Finnish adolescents.

Periodontal diseases are diagnosed mainly by clinical and radiological examinations, which shows only the past, not the current situation, much less the future. Oral fluids contain many biomarkers, such as aMMP-8, PGLYRP1, TREM-1, and PMN elastase, all of which are associated with periodontal diseases. Thus, monitoring salivary biomarkers for oral diseases should complement clinical examinations.

Elevated salivary levels of PGLYRP1 in adolescents with gingivitis and subclinical periodontitis and its positive correlation with TREM-1 and aMMP-8 may indicate an association of PGLYRP1 with subclinical periodontitis. Poor oral hygiene is also associated with higher levels of PGLYRP1. However, salivary PGLYRP1 might have a lower discriminating capacity, therefore being a less reliable marker alone in the diagnosis of periodontal disease in adolescents.

The aMMP-8 chairside mouth rinse test could identify and alert adolescents with poor oral hygiene at risk for subclinical periodontitis without visible manifestations of illness such as deepened periodontal pockets. Thus, young at-risk patients could be recognized and allocated to preventive intervention at a very early stage of the periodontal disease. The aMMP-8 chairside mouth rinse test become negative over follow-up with improved periodontal health parameters. The test has an ability to identify active periods of periodontitis in real-time.

More attention should be paid to sufficient preventive intervention, early diagnosis, and treatment of adolescents with early signs of periodontal oral disease. Periodontal diseases have a large-scale impact. Periodontal diseases are very common and costly, not to mention being linked to systemic diseases such as diabetes.

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